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1. AGENCY USE ONLY (Leave blank)

2. REPORT DATE  
1991

3. REPORT TYPE AND DATES COVERED

Annual: 01 Dec 88 To 01 Mar 89

4. TITLE AND SUBTITLE

Biodegradation of Polychlorinated Methanes in  
Methanogenic Systems

5. FUNDING NUMBERS

G- AFOSR-89-0189

AF- 61102F

PR- 2312

TA- A5

(2)

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REPORT NUMBER

AFOSR-TR- 92 0648

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Department of the Air Force  
Air Force Office of Scientific Research  
Directorate of Life Sciences  
Bolling Air Force Base, DC10. SPONSORING/MONITORING  
AGENCY REPORT NUMBER

NL

11. SUPPLEMENTARY NOTES

Fellowship awarded through the American College of Toxicology, 9650 Rockville Pike,  
Bethesda, MD 20814

12a. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release;  
distribution unlimited.

92 7 20 163

12b. DISTRIBUTION CODE

DTIC  
ELECTE  
JUL 21 1992  
S A D

13. ABSTRACT (Maximum 200 words)

This research investigated biodegradation of several halogenated methanes under methanogenic conditions. Biodegradation of dichloromethane to CO<sub>2</sub> and acetic acid (both environmentally acceptable products) was demonstrated in a fixed-film reactor, operated at 20°C, a residence time as low as 0.25 day, and an influent concentration of 91 µM. The biodegradability of chloroform was examined in a dichloromethane-degrading enrichment culture. Sustained consumption of chloroform (approximately 8.5 µM) was achieved only when vitamin B<sub>12</sub> was also added. Initially, equimolar amounts of chloroform and B<sub>12</sub> were added; when the amount of B<sub>12</sub> added was gradually decreased to zero, chloroform degradation continued at the same rapid rate. Biodegradation of <sup>14</sup>C-chloroform yielded approximately 77% CO<sub>2</sub>, 10% carbon monoxide, 7% soluble compounds (about 15% of which consisted of acetate), and 2% nonsoluble compounds; no <sup>14</sup>CH<sub>4</sub> was formed. Brominated methanes (dibromomethane, bromochloromethane, and bromomethane) degraded much more slowly, if at all, than dichloromethane; they inhibited methanogenesis and dichloromethane degradation. Sustained degradation of chloromethane was demonstrated for an extended period without the need for an electron donor. Preliminary results suggest chloromethane serves as an electron donor under methanogenic conditions, just as dichloromethane does. Biotransformation of carbon tetrachloride was also demonstrated in the dichloromethane-degrading enrichment culture.

14. SUBJECT TERMS

Biodegradation; methanogenic systems; chlorinated solvents;  
dichloromethane; chloroform; carbon tetrachloride; fixed-film

15. NUMBER OF PAGES

16. PRICE CODE

17. SECURITY CLASSIFICATION  
OF REPORT

Unclassified

18. SECURITY CLASSIFICATION  
OF THIS PAGE

Unclassified

19. SECURITY CLASSIFICATION  
OF ABSTRACT

Unclassified

20. LIMITATION OF ABSTRACT

UL

NSN 7540-01-280-3500

Standard Form 298 (Rev. 2-89)  
Prescribed by ANSI Std. Z39-18

23 AUG 1991

## I: STATEMENT OF WORK

### A. Objectives

The broadly defined objective of this research is to examine the fundamental factors influencing biodegradation of polyhalogenated methanes in methanogenic systems. This relates directly to the AFSC's interest in reducing the risk of bioenvironmental hazards posed by compounds used in Air Force activities. The specific objectives of this research are:

1) To Isolate the microorganisms directly responsible for biodegradation of dichloromethane (DCM) under methanogenic conditions. The pure cultures will then be characterized, particularly with regard to physiological properties. Cell-free extracts will also be used to make a detailed analysis of the DCM degradation pathways. In the absence of obtaining an isolate, more information of degradation pathways will be sought using selective enrichments.

2) To evaluate the ability of DCM-degrading enrichment cultures to degrade halogenated compounds other than DCM. This includes other C<sub>1</sub> compounds, as well as at least one C<sub>2</sub> chlorinated aliphatic such as trichloroethylene.

3) To examine the ability of DCM-degrading enrichment cultures to biodegrade chloroform (CF) and carbon tetrachloride (CT) under methanogenic conditions. If transformation of CF and CT is demonstrated, [<sup>14</sup>C]CF and [<sup>14</sup>C]CT will be used to determine the degradation products.

### B. Background

Prior to this project, Dr. Freedman conducted research on biodegradation of DCM under methanogenic conditions. The research presented in this report expands on several elements of the preceding work. Thus, in order to understand the current results, a summary of the major findings from the previous studies with DCM is presented below. In addition, Appendix A contains a copy of a paper (accepted for publication in *Applied and Environmental Microbiology*) describing in more detail the previous work with DCM.

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Biodegradation of DCM to environmentally acceptable products —  $\text{CO}_2$ ,  $\text{CH}_4$ , and acetic acid — was demonstrated under methanogenic conditions ( $35^\circ\text{C}$ ). When DCM was supplied to enrichment cultures as the sole organic compound, at a low enough concentration to avoid inhibition of methanogenesis, the molar ratio of  $\text{CH}_4$  formed to DCM consumed (0.473) was very close to the amount predicted by stoichiometric conservation of electrons. DCM degradation was also demonstrated when methanogenesis was partially inhibited (with 0.5-1.5 mM 2-bromoethanesulfonate or approximately 2 mM DCM) or completely stopped (with 50-55.5 mM 2-bromoethanesulfonate). Addition of a eubacterial inhibitor (vancomycin, 100 mg/liter) greatly reduced the rate of DCM degradation.  $^{14}\text{CO}_2$  was the principal product of [ $^{14}\text{C}$ ]DCM degradation, followed by  $^{14}\text{CH}_4$  (when methanogenesis was uninhibited) or  $^{14}\text{CH}_3\text{COOH}$  (when methanogenesis was partially or completely inhibited). Hydrogen accumulated during DCM degradation, then returned to background levels when DCM was consumed.

These results were used to develop a model of DCM degradation (Figure 1; also described in Appendix A). The organisms mediating DCM oxidation to  $\text{CO}_2$  were termed "DCM oxidizers." The other major mode of DCM biotransformation — fermentation to acetic acid — was likely mediated by acetogenic bacteria. The methyl carbon of the acetate formed came directly from DCM and the carboxyl carbon came from unlabeled  $\text{CO}_2$ , available in the large pool of carbonates present in the basal medium. DCM degradation is thus a disproportionation: a portion of the DCM is oxidized, making reducing equivalents available for reduction of an equal amount of DCM.

Methanogens consumed the products of DCM degradation.  $\text{CO}_2$ -reducing methanogens used some of the electrons made available from DCM oxidation to form methane. At least a portion of those electrons were available in the form of  $\text{H}_2$ . Methane produced by  $\text{CO}_2$ -reducers was unlabeled because the  $^{14}\text{CO}_2$  formed from oxidation of  $^{14}\text{CH}_2\text{Cl}_2$  was essentially diluted out by the large unlabeled carbonate pool in the basal medium. Aceticlastic methanogens produced methane from the acetic acid formed by fermentation of DCM.

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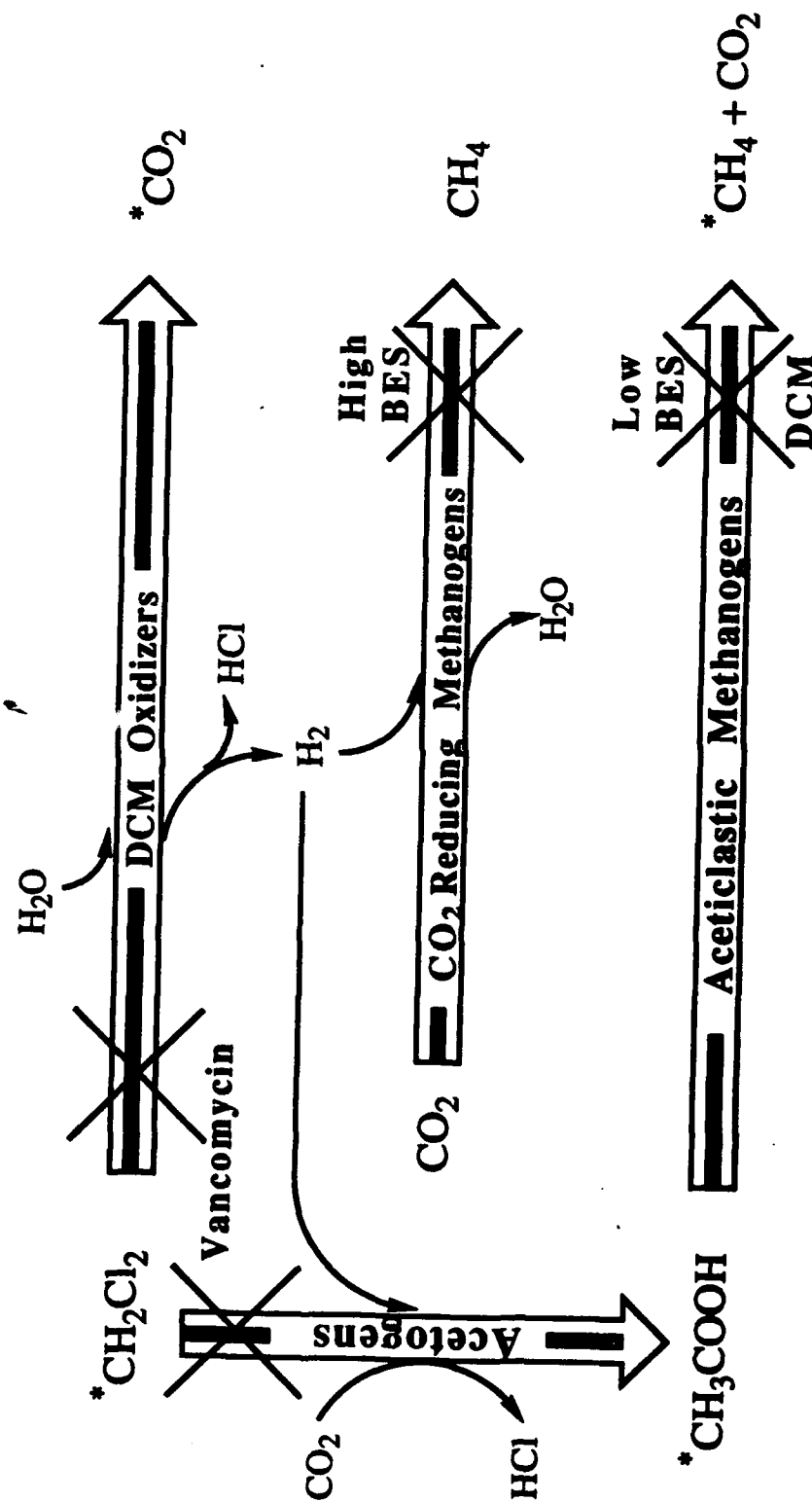


Figure 1: Proposed model for biodegradation of DCM under methanogenic conditions.

Figure 1 also indicates where inhibitors exerted their effects. DCM degradation was virtually stopped by vancomycin. Aceticlastic methanogens were inhibited by low doses of 2-bromoethanesulfonate (0.5-1.5 mM) or DCM additions above approximately 0.15 mM. Significant inhibition of CO<sub>2</sub>-reducing methanogens was observed only with very high doses of 2-bromoethanesulfonate (50 mM).

One important aspect of DCM degradation under methanogenic conditions not shown in Figure 1 is the ability of enrichment cultures to use this halogenated aliphatic compound as a growth substrate. When DCM was provided as the sole organic-carbon and electron-donor source, the highest observed yield was 0.085 grams of suspended organic carbon formed per gram of DCM carbon consumed. Approximately 85% of the biomass formed was attributable to the growth of nonmethanogens, 15% to methanogens.

This project is making use of the previously developed enrichment culture, which has served as the starting point for isolation of the DCM-degrading organisms and evaluation of the biodegradability of structurally similar halogenated aliphatic compounds.

## **II: STATUS OF THE RESEARCH**

### **A. Studies with DCM**

#### **1. DCM Degradation in a Fixed-Film Reactor**

This part of the research was an extension of work funded by the U. S. Air Force Engineering and Services Center, Tyndall AFB, Florida, under contract no. F08635-86-C-0161. The purpose was to examine DCM degradation in a fixed-film reactor that simulated treatment of DCM-contaminated groundwater, either in an above-ground reactor or in situ. Results from this study were presented at *"In Situ and On-Site Bioreclamation — An International Symposium,"*

sponsored by Battelle, Inc. A copy of the paper submitted is contained in Appendix B; it will be published this fall in the Symposium Proceedings. A summary of the findings is presented below.

The feasibility of biodegrading DCM to environmentally acceptable products was demonstrated in a fixed-film reactor operated under methanogenic conditions. Two glass columns (51-mm i.d. x 914-mm, filled with 6-mm glass beads; void volume = 730 mL) connected in series comprised the reactor. The sole purpose of the first column was to reduce the redox potential of the feed to the second column. For 14 months, the second column received the anaerobic effluent from the first column plus DCM (as saturated water), the only major source of carbon and energy added.

Initial operation was conducted at 35°C, a 2.0-day hydraulic retention time, and a DCM influent concentration of 100  $\mu\text{M}$ ; nearly 99% of the DCM was degraded between the influent sampling port and the first column port (spaced every 152-mm). The temperature was lowered to 20°C and the influent DCM concentration was gradually increased until breakthrough occurred at approximately 780  $\mu\text{M}$  (11.9  $\mu\text{mol DCM/hr}$ ). Concurrent with the appearance of DCM in the effluent was a sharp reduction in total methane output, indicating inhibition of methanogenesis at all levels of the second column. The hydraulic retention time of the reactor was gradually lowered from 2.0-d to 0.25-d, and maintained at this level for 34 days. Complete DCM removal was achieved at an influent concentration of 91  $\mu\text{M}$  (11.0  $\mu\text{mol DCM/hr}$ ), while breakthrough occurred at 130  $\mu\text{M}$  (15.8  $\mu\text{mol DCM/hr}$ ).

The fate of DCM was examined by continuous addition of [ $^{14}\text{C}$ ]DCM, when the hydraulic retention time was at 0.25-d. Approximately two thirds of the label was recovered as  $^{14}\text{CO}_2$  and one third as a nonvolatile compound, most of which was shown to be acetic acid. These results were consistent with [ $^{14}\text{C}$ ]DCM experiments that utilized batch enrichment cultures.

## **2. Steps Towards Isolation of the DCM-Degrading Organism(s)**

The strategy employed to isolate the DCM-degrading organisms has been serial dilution of an enrichment culture that actively degrades DCM. A question arose early in this work

with regard to the substrate that should be used during serial dilution. While DCM may seem to be the logical choice, its use has at least two disadvantages. First, the DCM-degrading organisms grow relatively slowly on DCM. For example, a culture starting with a 10% inoculum of an enrichment culture needed 77 days to increase its cell concentration by 50 mg/L. This is due in part to the low growth yield, the highest observed value being 0.060 grams of cells (dry weight organic matter) formed per gram of DCM oxygen demand consumed [3]. With serial dilutions, the amount of inoculum would be orders of magnitudes lower than 10%, so presumably it would take an extremely long time (i.e., months, or possibly years) for the DCM-degraders to grow.

The second problem with using DCM as a substrate in serial dilutions is that it appears to be inhibitory. Thus far, the highest level of DCM added to enrichment cultures without signs of inhibition has been approximately 4.4 mM. Two cultures have received this level and degraded it within three days. Why inhibition occurs above 4.4 mM is unclear. One possible reason is that a large amount of DCM-saturated water is needed (more than 2.0 mL) to add more than 440  $\mu$ mol of DCM to 100 mL of culture (in a 160 mL serum bottle). The DCM-saturated water contains some amount of oxygen in it; when more than 2.0 mL are added to a culture, the redox level may rise above a critical level. To avoid this, additions of neat DCM have been tried, since the volume required to achieve the same mass is far lower. However, neat additions have consistently resulted in greatly reducing the rate of DCM degradation, for unknown reasons. Work is now underway to try to raise the DCM additions above 4.4 mM using DCM-saturated basal medium, which will maintain a low redox potential in the resulting solution. However, the possible abiotic affect of low redox conditions on neat DCM in basal medium is not yet known.

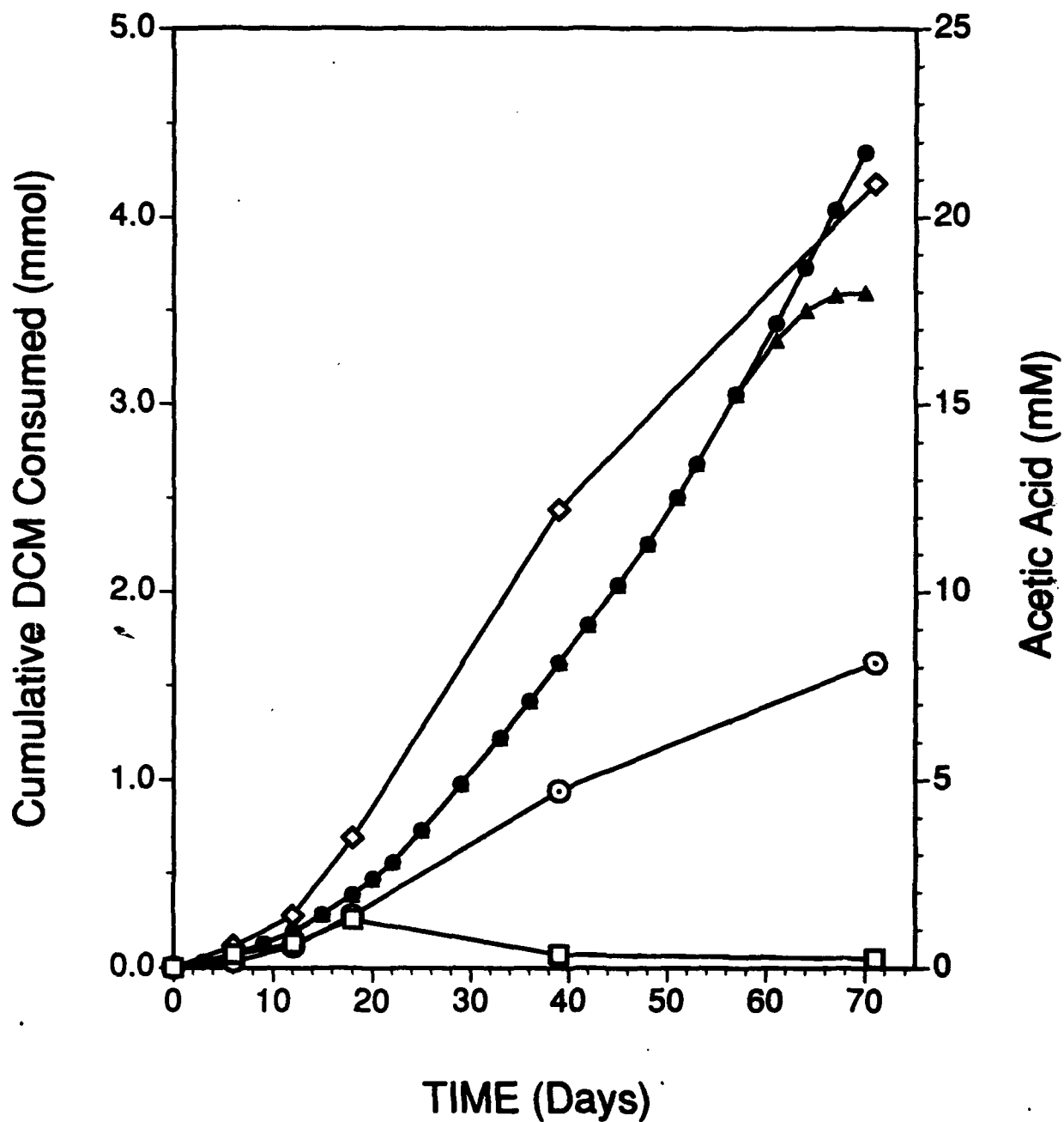
Given the difficulties of using DCM as a substrate in serial dilutions, an alternate approach was explored. The intent was to use a substrate other than DCM that supports a much more rapid rate of growth of DCM-degrading organisms, and one that is not inhibitory. Once the culture has been grown up on the alternate substrate, it would be switched back to DCM. Preliminary experiments have been done with pyruvate, since the accumulated evidence thus far indicates that

acetogenic bacteria mediate DCM degradation, and virtually all known acetogens use pyruvate. Another possible advantage of using pyruvate is that methanogenic bacteria (the other major group of bacteria evident in DCM-degrading enrichment cultures) cannot metabolize it. Six serum bottle cultures were tested; all were started with 100 mL from the same DCM-degrading culture. Two received only DCM additions; two received DCM plus an equivalent amount of pyruvate on a chemical oxygen demand (COD) basis (i.e., approximately 0.4 mol pyruvate per mol DCM); and two received only pyruvate, added at the same rate of COD as the DCM-only bottles.

The amount of DCM added was gradually increased from an initial dose of 30  $\mu\text{mol}$  per bottle to 310  $\mu\text{mol}$  per bottle every third day, by day 70. The concentration of acetic acid and pyruvate was periodically monitored using HPLC. Results are shown in Figure 2. Through day 57, the rate of DCM degradation was the same in cultures with and without pyruvate added; thereafter, DCM degradation in the bottles with pyruvate slowed down. However, it is unclear if that was a consequence of the pyruvate, or the extra amount of non-reduced solution added to the cultures (i.e., the pyruvate stock solution was dissolved in water). Acetate accumulation was greatest in the cultures receiving DCM plus pyruvate, followed by those receiving only DCM. Only a slight amount of acetate accumulated in the pyruvate only bottles; all of the pyruvate added was consumed. Acetogens degrade pyruvate to acetate,  $\text{CO}_2$  and hydrogen; DCM is degraded to the same products. Earlier work demonstrated that DCM additions above 15  $\mu\text{mol}$  per bottle inhibits aceticlastic methanogens (but not  $\text{CO}_2$ -reducing methanogens); this accounts for the accumulation of acetic acid in the bottles receiving DCM. Methane output (data not shown) was highest in the bottles receiving only pyruvate, even though they received less COD than the DCM plus pyruvate bottles.

All six bottles were periodically examined by phase contrast microscopy. In the bottles receiving only DCM, the predominant morphology was a slightly bent non-motile rod; often these occurred in short chains. The bottles receiving only pyruvate contained numerous highly motile cocci, but no rods. Those receiving DCM plus pyruvate contained a mixture of rods and motile





**Figure 2:** Biodegradation of DCM in cultures receiving (▲) and not receiving (●) pyruvate, and acetic acid formation in cultures receiving DCM plus pyruvate (◆), DCM only (○), and pyruvate only (□).

cocci. Thus, the organisms most likely mediating DCM degradation (the bent rods) were not enriched for by the presence of pyruvate, making it a poor choice of substrates for isolation of the DCM-degraders. Other substrates may be tried under similar conditions. Subsequent tests will also examine the ability of bottles not receiving DCM to start degrading it. This is of concern because previous work has suggested that when the DCM-degrading organisms go without DCM for extended periods (e.g., greater than one week), there is a long lag period before the ability to rapidly degrade DCM returns.

In order to make the isolation process somewhat easier, work was undertaken to transfer the DCM-degrading culture to a more "user-friendly" basal medium. The problem with the original medium is that it contains a high amount of iron and sodium sulfide, resulting in formation of iron sulfides (Table 1). The black iron sulfides make it impossible to visually determine if cell mass is increasing. Furthermore, the precipitates make microscopic examination of the culture more distracting. The new medium contains far less iron and enough nitrilotriacetic acid to chelate the metals; it is transparent and slightly yellow. As a consequence, accumulation of cell mass is easy to detect visually, and microscopic examination is much improved. Transfer of the DCM-degrading enrichment culture to the new medium has been successful. The only drawback is that the new medium appears to have less redox buffering capacity; it occasionally turns pink after purging the headspace of accumulated methane, then returns to a transparent color. The new medium will be used in serial dilutions aimed at isolating the DCM-degrading organism.

If DCM is used as the substrate in serial dilutions, one concern is the initial amount that can be added. As mentioned above, DCM appears to be an inhibitory substrate. If the inhibitory affect is related to biomass concentration as well as DCM concentration, then the greater the dilution, the greater the possibility that DCM will prevent growth. Because of this concern, new cultures have typically been started with a low dose of DCM — on the order of 10-30  $\mu\text{mol}$  per bottle, depending on the size of the inoculum ( $\geq 2\%$ ). Once degradation is observed, the amount of DCM repetitively added is gradually increased, thereby avoiding any possible inhibition of the DCM-

**Table 1:**  
**Basal Media for DCM-Degrading Enrichment Cultures**

Component	Original Medium (Quantity/L)	New Medium (Quantity/L)
NH <sub>4</sub> Cl	0.20 g	0.50 g
K <sub>2</sub> HPO <sub>4</sub> ·3H <sub>2</sub> O	0.10 g	0.524 g
KH <sub>2</sub> PO <sub>4</sub>	0.055 g	-
MgCl <sub>2</sub> ·6H <sub>2</sub> O	0.20 g	0.10 g
Trace metal solution	10 mL <sup>a</sup>	10 mL <sup>b</sup>
Na <sub>2</sub> S·9H <sub>2</sub> O	0.50 g	0.50 g
FeCl <sub>2</sub> ·4H <sub>2</sub> O	0.10 g	-
NaHCO <sub>3</sub>	5.0 g	4.0 g
CaCl <sub>2</sub> ·2H <sub>2</sub> O	-	0.10 g
Yeast Extract	0.050 g	0.050 g
Resazurin	1 mg	1 mg

<sup>a</sup> 0.10 g/L MnCl<sub>2</sub>·4H<sub>2</sub>O; 0.17 g/L CoCl<sub>2</sub>·6H<sub>2</sub>O; 0.10 g/L ZnCl<sub>2</sub>; 0.20 g/L CaCl<sub>2</sub>; 0.019 g/L H<sub>3</sub>BO<sub>4</sub>; 0.050 g/L NiCl<sub>2</sub>·6H<sub>2</sub>O; and 0.020 g/L Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O. Adjusted to pH 7 with NaOH.

<sup>b</sup> 0.10 g/L MnCl<sub>2</sub>·4H<sub>2</sub>O; 0.17 g/L CoCl<sub>2</sub>·6H<sub>2</sub>O; 0.10 g/L ZnCl<sub>2</sub>; 0.020 g/L CaCl<sub>2</sub>; 0.019 g/L H<sub>3</sub>BO<sub>4</sub>; 0.050 g/L NiCl<sub>2</sub>·6H<sub>2</sub>O; 0.020 g/L Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O; 0.4 g/L FeCl<sub>2</sub>·4H<sub>2</sub>O; and 4.5 g/L nitrilotriacetic acid. Adjusted to pH 7 with NaOH.

degraders. An experiment was set-up to examine the use of a much higher initial dose of DCM, with varying levels of inoculum. Nine bottles were inoculated with a culture that was degrading 300  $\mu$ mol DCM per bottle within three days. Three received 10.0 mL of inoculum, three received 1.0 mL, and three received 0.1 mL; the balance of the 100 mL of culture was basal medium. All were given approximately 120  $\mu$ mol DCM on day 0.

Degradation of the initial DCM dose was achieved in all of the bottles; within 100 days in the bottles with 10 mL inoculum, 135 days in the bottles with 1.0 mL inoculum, and 175 days in the bottles with 0.1 mL inoculum. Methanethiol and a number of unidentified peaks (excluding chloromethane) appeared in all of the bottles during routine headspace analysis by gas chromatography (GC) with a flame ionization detector; these compounds gradually disappeared once the DCM was degraded. These results suggest that bottles set-up with a low level of inoculum (as required in a serial dilution, e.g.,  $10^{-8}$  mL) stand a chance of degrading a relatively high initial addition of DCM.

## **B. Studies with Other Di- and Monohalogenated Methanes**

The initial interest in examining the ability of DCM-degrading enrichment cultures to metabolize other dihalogenated compounds was based on a report by Bowser and Wright [1]. They indicated that brominated aliphatics are more readily degraded than chlorinated ones. If this were so for dihalomethanes, then use of dibromomethane (DBM) rather than DCM might make isolation more easy, especially if DBM proved to be less inhibitory at high concentrations. Furthermore, it was envisioned that switching cultures from DBM to DCM could be done without a lag, an issue of concern when trying to grow the DCM-degrading organisms with a nonhalogenated substrate. Experiments were therefore undertaken with DBM, and subsequently with bromochloromethane (BCM), chloromethane (CM), and bromomethane (BM).

Analysis of each of these compounds was the same as for DCM: The total mass present in a serum bottle (160 mL total volume, filled with 60 mL of culture) was determined by measuring

the response of a GC with a flame ionization detector to an injection of a 0.5 mL headspace sample. Response factors for each compound were obtained by adding known amounts to serum bottles containing 100 mL of distilled deionized water, equilibrating at 35°C in an orbital water bath, then injecting 0.5 mL of the headspace onto the GC. CM and BM were added as gases.

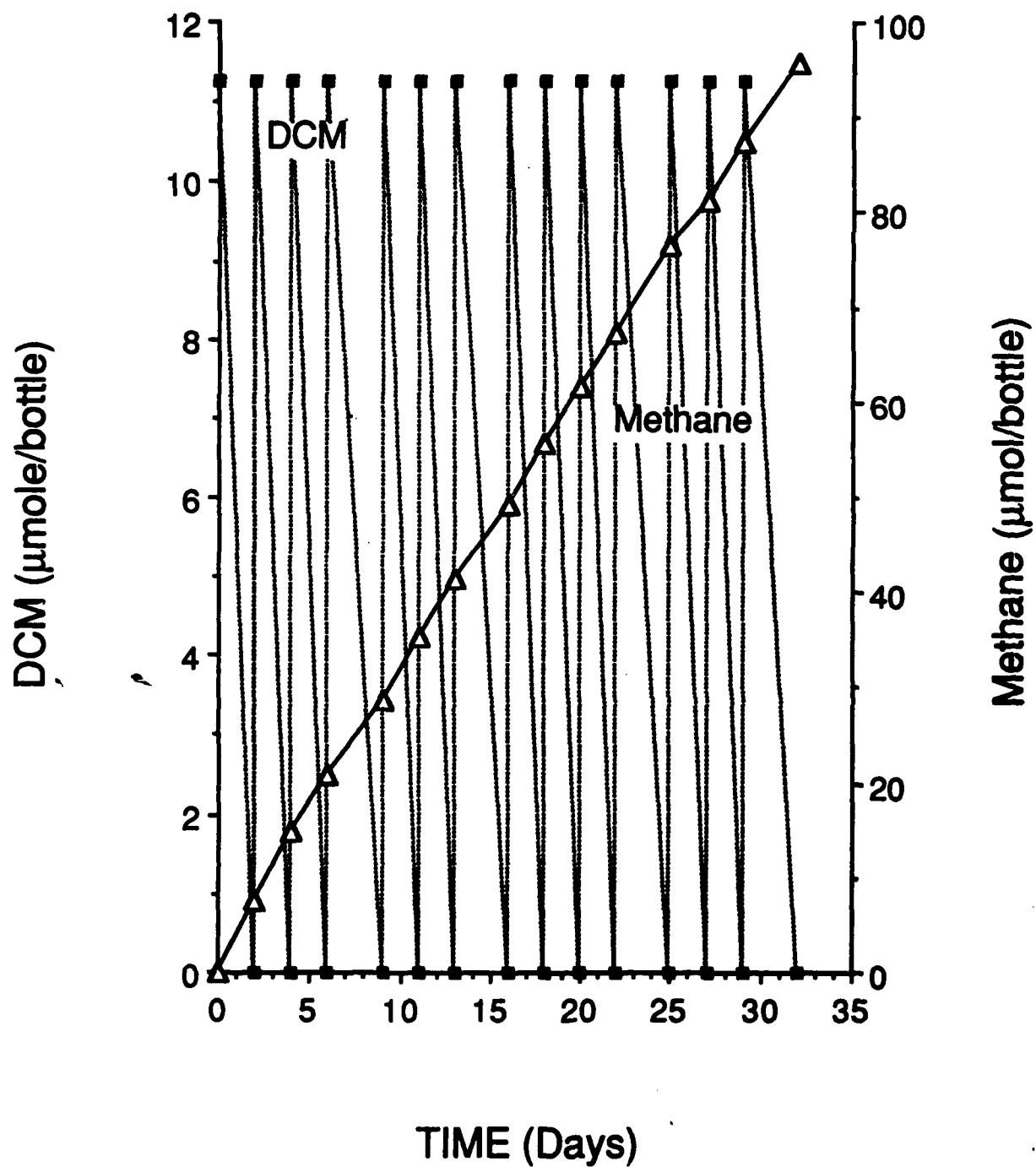
In all experiments described below, serum bottles were started with 100 mL of culture from a 2-L reactor that degraded approximately 30  $\mu\text{mol}$  of DCM per 100 mL of culture within three days. Culture from the 2-L reactor was dispensed to serum bottles in an anaerobic glove box, containing an atmosphere of 99.0-99.7%  $\text{N}_2$ , 0.3-1.0%  $\text{H}_2$ . Once the transfer was made, the serum bottles were stoppered with slotted grey-butyl septa (except where noted), held in place with aluminum crimp caps. Outside of the glove box, they were purged for about two minutes with 30%  $\text{CO}_2$ -70%  $\text{N}_2$  and recapped; purging was necessary to remove the  $\text{H}_2$  (a potential electron donor) and balance the headspace  $\text{CO}_2$  with the basal medium bicarbonate (to maintain a culture medium pH of about 7).

#### 1. Biodegradation of Dibromomethane

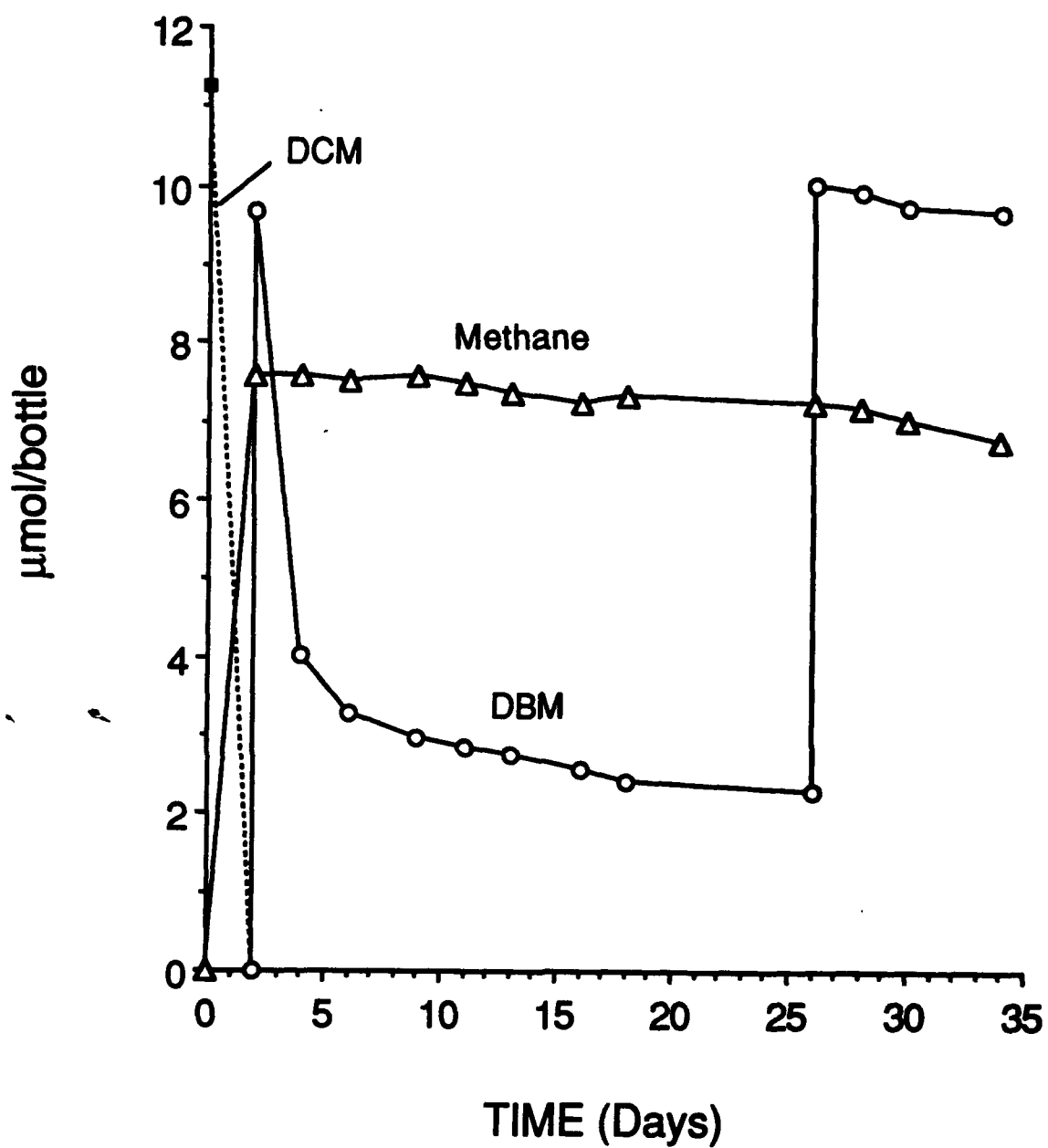
Biodegradation of DBM was evaluated using six bottles. Before adding DBM, each bottle received approximately 11  $\mu\text{mol}$  of DCM immediately after the transfer from the 2-L reactor. This was done to check that the transfer did not interfere with the culture's ability to continue degrading DCM.

After degrading the initial dose of DCM, two bottles — serving as controls — continued to receive 10-12  $\mu\text{mol}$  of DCM every other day. As shown in Figure 3, the bottles encountered no difficulty in repetitively degrading DCM, resulting in a nearly stoichiometric output of methane.

After degrading the initial dose of DCM, two of the bottles received approximately 10  $\mu\text{mol}$  of DBM on day 2. The DBM concentration declined quickly at first, then leveled off. Even after more DBM was added on day 26, very little was degraded. The presence of DBM resulted in total inhibition of methane production (Figure 4).



**Figure 3:** Typical performance of a control bottle degrading DCM, with nearly stoichiometric formation of methane.



**Figure 4:** Biodegradation of DBM, following degradation of an initial dose of DCM.

The remaining two bottles were used to determine if the DCM-degrading culture would adapt to use of DBM by gradually reducing the amount of DCM added and using a lower initial dose of DBM. As shown in Figure 5, this is not what happened. By day 2, the initial 11  $\mu\text{mol}$  of DCM supplied was degraded and 9  $\mu\text{mol}$  of DCM was added along with 2  $\mu\text{mol}$  of DBM. Most of the DBM was degraded in two days, but the rate of DCM degradation was noticeably slower. The next addition of DBM declined by only 1.2  $\mu\text{mol}$  between day 6 and day 26, and the final DBM addition was depleted even more slowly. Coinciding with a decrease in the rate of DBM degradation was a complete stop in DCM degradation and methane production (Figure 5). Thus, contrary to expectations, DBM turned out to be a poor substrate for the DCM-degraders, and caused a complete inhibition of DCM degradation as well as methanogenesis.

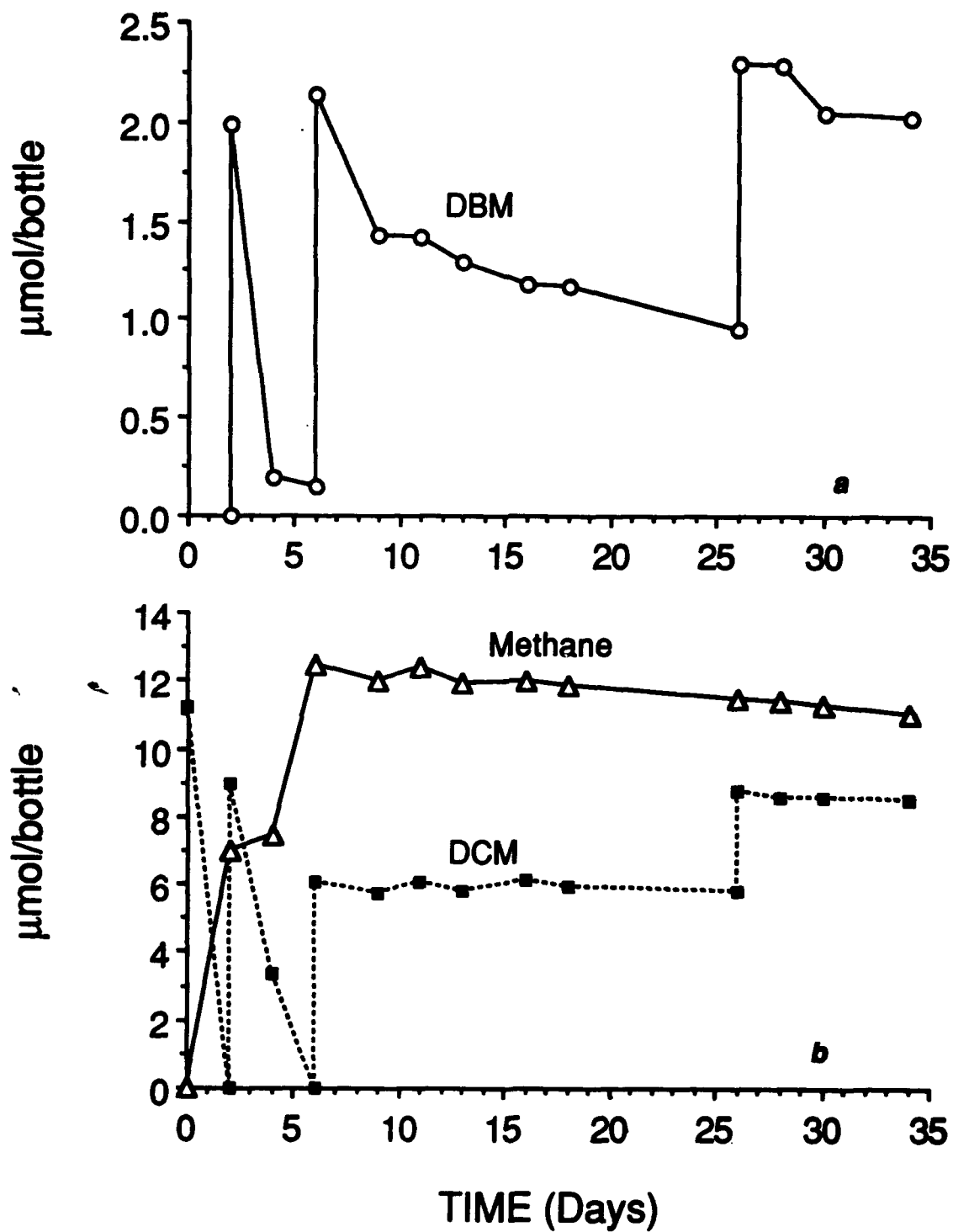
Both Figures 4 and 5 suggest that methanogenesis was inhibited by DBM. An alternative explanation for the cessation of methane output might be the lack of any electron donors; i.e., if DBM and DCM were not degrading, how could the methanogens receive any substrate (via acetate or hydrogen)? Two bottles were set-up to address this. Both received 100 mL of culture from the 2-L reactor and nothing else. As shown in Figure 6, methane was produced well beyond days 2-6, when it stopped in the bottles receiving DBM. The source of electron donor for methane production in cultures taken from the 2-L reactor was not identified. However, low levels of acetate (from DCM degradation) and yeast extract are likely possibilities. Measurements of acetate and COD in the 2-L reactor culture will be made shortly.

DBM losses from duplicate water control bottles were minimal — averaging only 11.8% after 103 days of incubation.

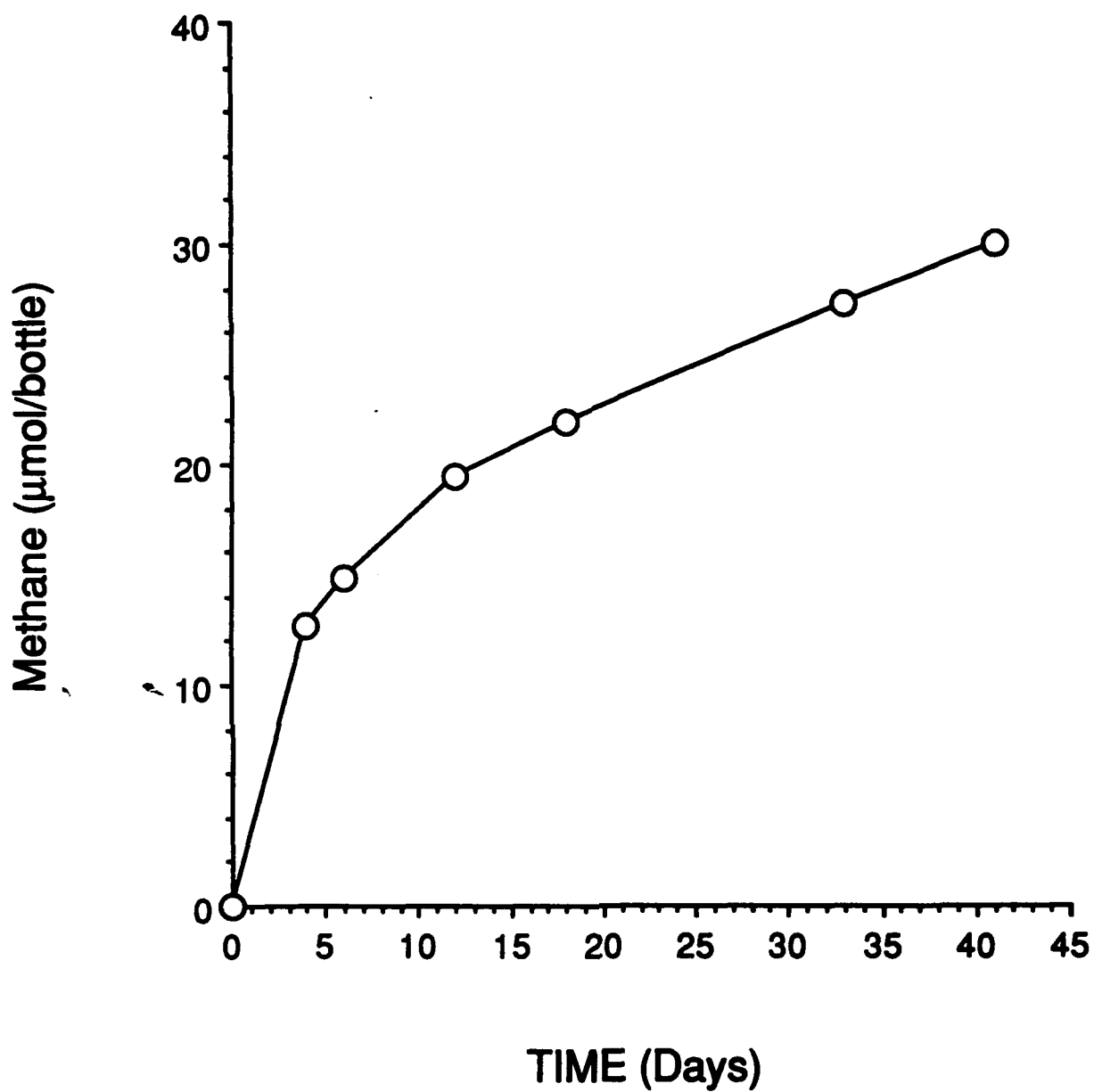
## **2. Biodegradation of Bromochloromethane**

The ability of the DCM-degrading enrichment cultures to biotransform another dihalomethane — BCM — was also tested. As with DBM, six bottles were set-up. Two served as controls, receiving only DCM; these bottles experienced no difficulty in repetitively degrading 10-12  $\mu\text{mol}$  additions every other day, resulting in nearly stoichiometric formation of methane. In





**Figure 5:** Biodegradation of low levels of DBM (a) while continuing additions of DCM in the same bottle (b).



**Figure 6:** Typical pattern of methane production in a serum bottle culture transferred from the 2-L reactor, when nothing else is added to the serum bottle.

two other bottles, the initial 12.5  $\mu\text{mol}$  of DCM was readily degraded, and methane increased as expected. However, addition of 12  $\mu\text{mol}$  of BCM on day 2 immediately stopped methane production, while the level of BCM fell very slowly (Figure 7). Even after 110 days of incubation, the BCM level did not fall below 2.7  $\mu\text{mol}$ , while complete methane inhibition continued.

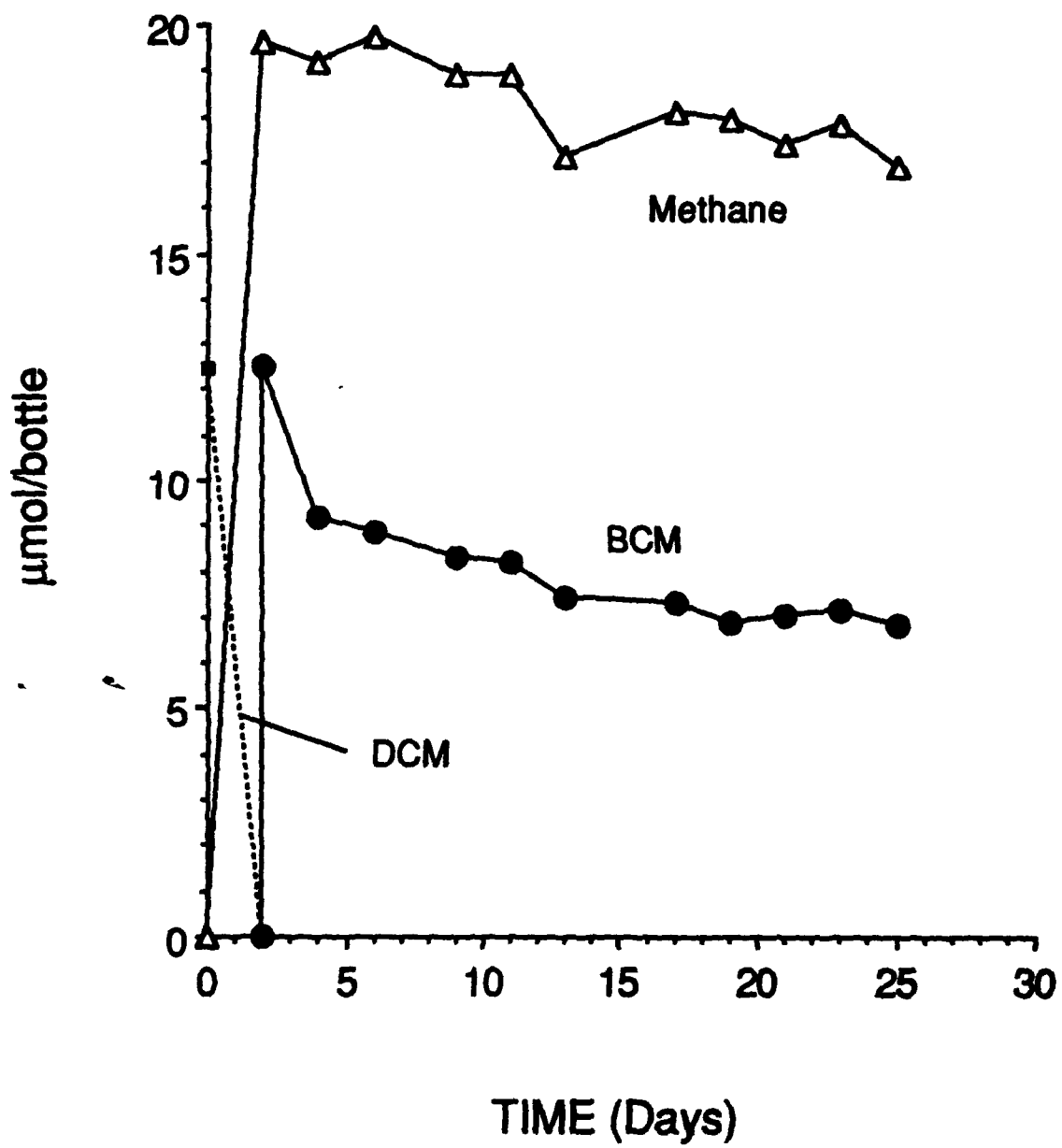
The last two bottles of this set received 2.6  $\mu\text{mol}$  BCM on day 2 along with 9  $\mu\text{mol}$  of DCM. Both the BCM and DCM were rapidly degraded, and methane output continued to rise. However, the second 2.8  $\mu\text{mol}$  addition of BCM was degraded more slowly, and caused a significant decrease in the rate of DCM degradation as well. Methane continued to accumulate, but very slowly (Figure 8). Even after 110 days of incubation, 0.65  $\mu\text{mol}$  of BCM remained in the bottle.

Thus, BCM also turned out to be a poor substrate for the DCM-degrading enrichment culture. Methanogenesis was completely inhibited by 10  $\mu\text{mol}$  BCM per bottle, as it was with a similar dose of DBM. The lower dose of BCM was only slightly more degradable and slightly less inhibitory to DCM-degraders and methanogens than the equivalent level of DBM.

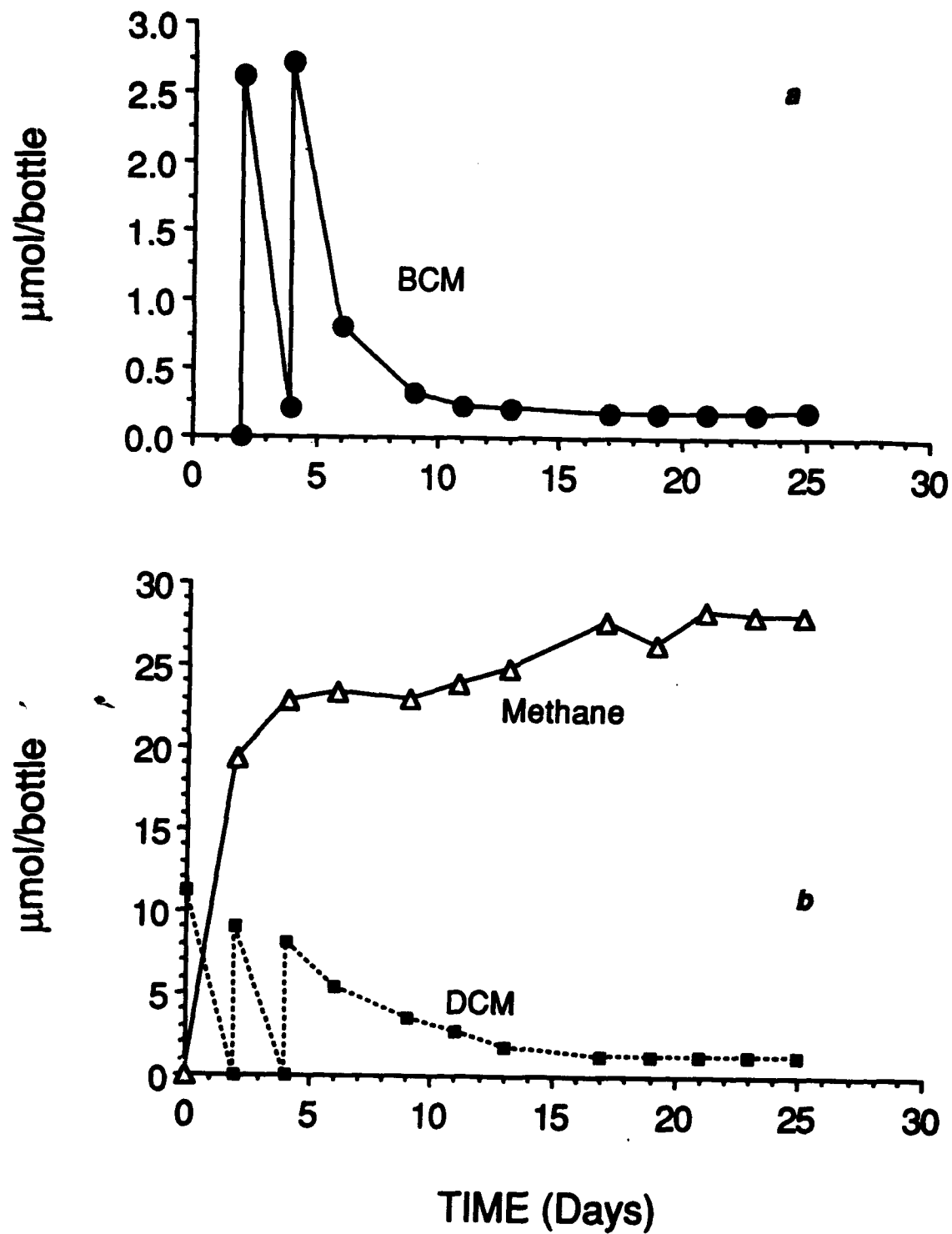
### 3. Biodegradation of Chloromethane

Experiments were undertaken with two monohalogenated methanes — CM and BM — to determine their potential usefulness as substrates for isolating the DCM-degrading organisms. As described above, culture from the 2-L reactor was transferred to six bottles; approximately 10  $\mu\text{mol}$  of DCM was added and degraded within two days. Two of the bottles then served as controls; they received repetitive DCM additions that were degraded every other day, and methane production was nearly stoichiometric.

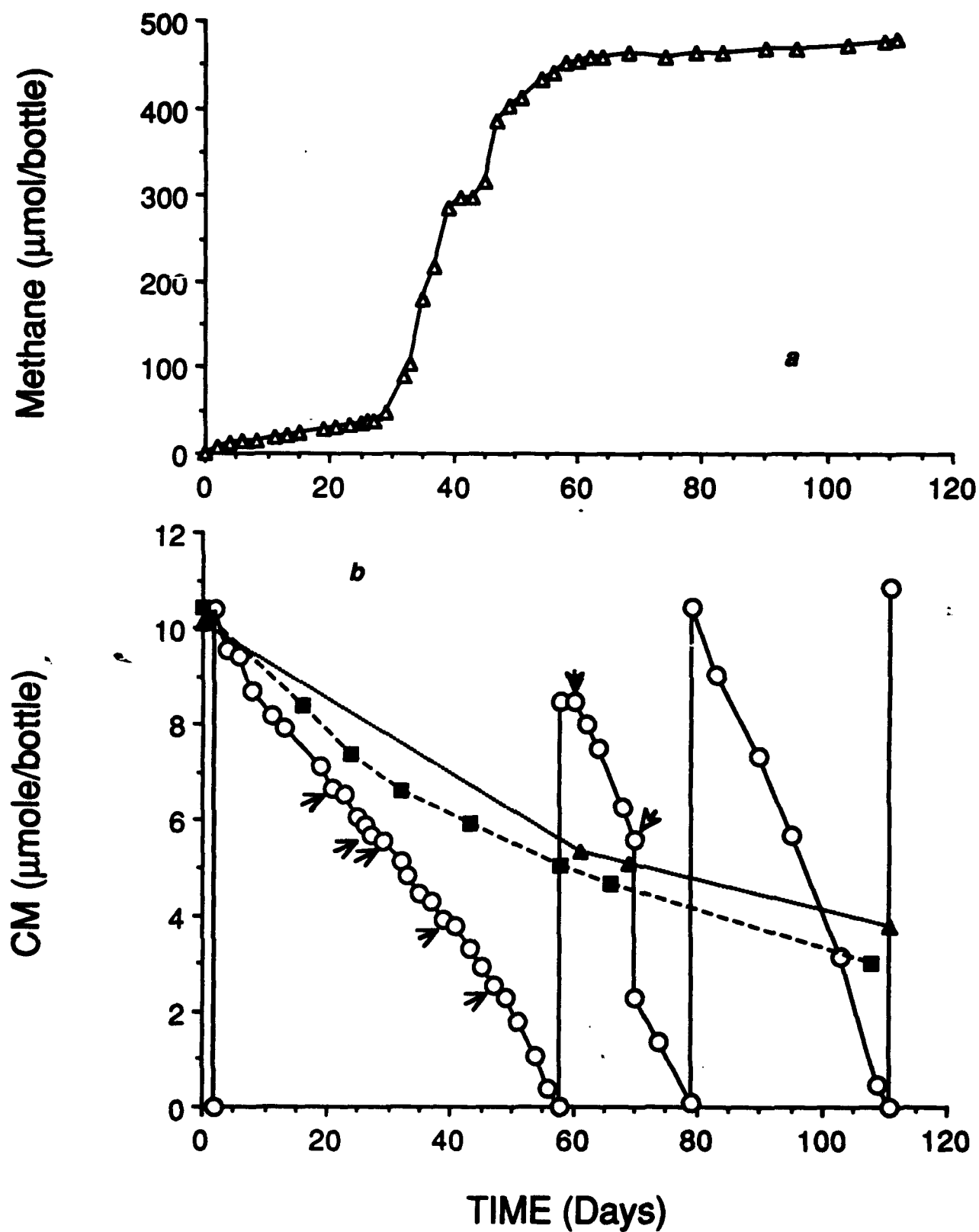
In two of the bottles, approximately 10  $\mu\text{mol}$  of CM was added on day 2. The rate of degradation was much slower than it had been for DCM. As shown in Figure 9, 58 days were required to degrade the first 10  $\mu\text{mol}$  addition of CM. Furthermore, the water and autoclaved-



**Figure 7:** Biodegradation of BCM, following degradation of an initial dose of DCM.



**Figure 8:** Biodegradation of low levels of BCM (a) while continuing additions of DCM in the same bottle (b).



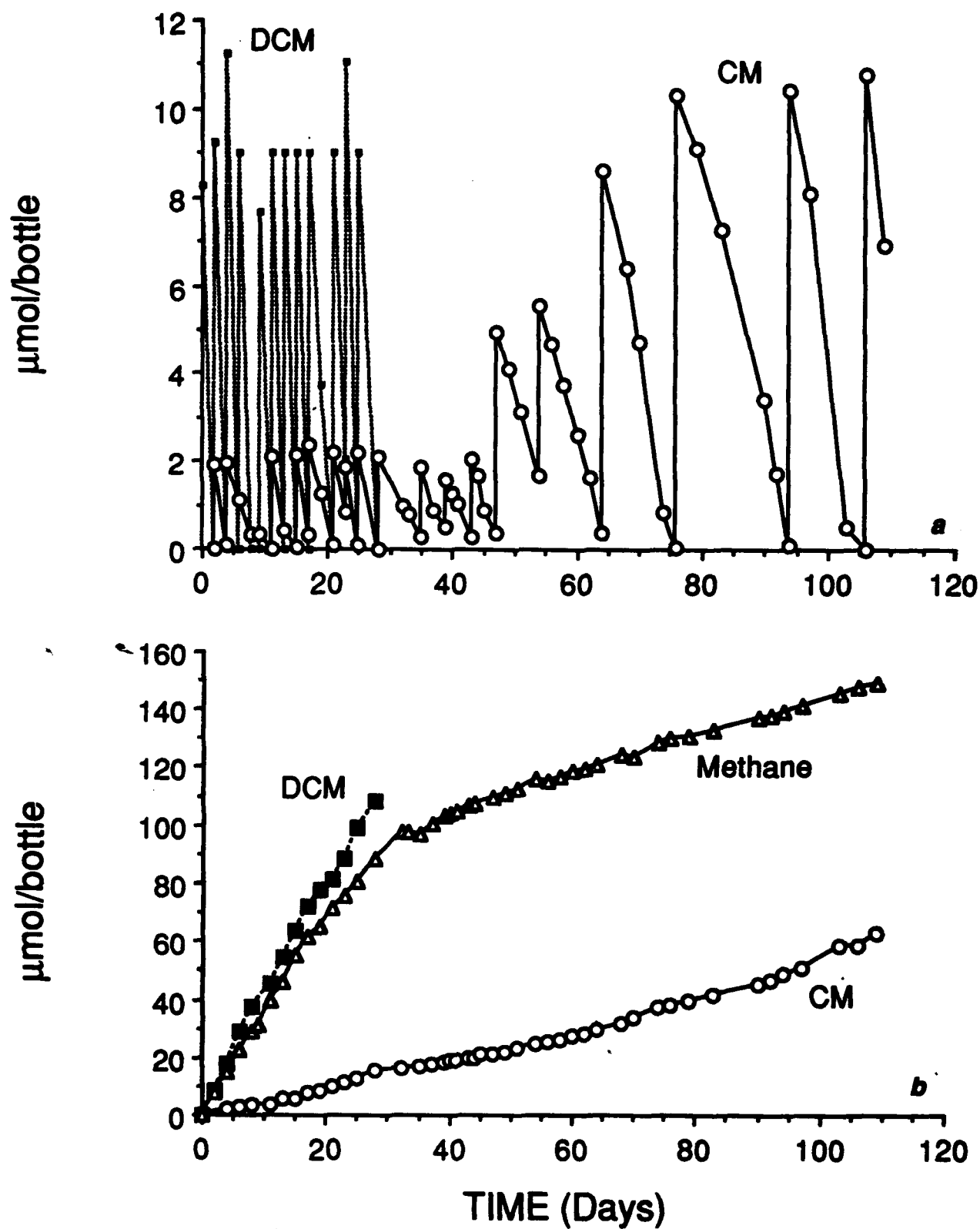
**Figure 9:** CM degradation in the inoculated bottle (O); for the inoculated bottle, arrows indicate methanol addition (↗), placement on a shake table (↓), and purging of the headspace (↖). CM in the water control (▲) and autoclaved-inoculated (■) bottles.

inoculated controls indicate that a portion of the CM disappearance was a consequence of loss through the bottles' septa.

An attempt was made to stimulate CM degradation in the inoculated bottles by addition of an electron donor, in the form of methanol. The bottles received five additions of 113  $\mu\text{mol}$  of methanol. Based on methane output between day 19 (first methanol addition) and day 60 (when output began to level off), conversion of methanol to methane was essentially stoichiometric. However, methane production did not respond until just after the third addition, and, more importantly, methanol did not alter the rate of CM degradation. On day 62, the two CM bottles were placed on a shake table, with the intent of improving the rate of CM degradation by agitating the cultures. This appears to have had a slight positive effect. On day 70 the bottles' headspaces were purged to remove the large amount of methane that had accumulated. Previous experience with these cultures has indicated that when methane is allowed to build up, DCM degradation is inhibited; however, the precise level at which this occurs is not yet known.

Operation of the two bottles described above is continuing. The rate of CM degradation appears to be gradually improving — only 32 days were required to degrade the third 10  $\mu\text{mol}$  addition of CM. If the rate continues to improve, the amount of CM added will be increased. The intent is to develop a CM enrichment culture, and then contrast it to the DCM-degrading cultures by microscopic examination.

The final two bottles used to examine CM degradation received only 2  $\mu\text{mol}$  on day 2 (instead of 10  $\mu\text{mol}$ ) and continued to receive 8-12  $\mu\text{mol}$  of DCM. As shown in Figure 10a, these cultures readily degraded the DCM added within two days. Unlike DBM and BCM, CM did not inhibit DCM degradation. Repetitive 2  $\mu\text{mol}$  additions of CM were degraded within two to four days. At this point the question arose whether or not DCM degradation was sustaining CM degradation. This would be a reasonable scheme if some of the DCM degradation products — namely acetic acid and hydrogen — were required for reductive dechlorination of CM to methane. Beginning on day 28, DCM additions were stopped. Initially, CM degradation slowed down; 7

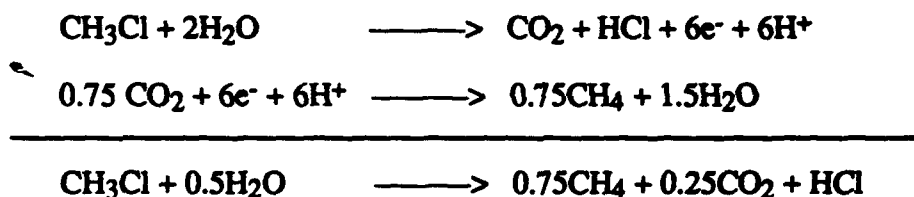


**Figure 10:** Simultaneous addition of CM and DCM, followed by CM additions only (a), and cumulative CM and DCM degradation, and  $\text{CH}_4$  formation in the same bottle (b).



days were required for consumption of 2  $\mu\text{mol}$ . However, subsequent CM additions were degraded within four days. Since day 47, the amount of CM added each time has been increased slightly. The overall rate of CM degradation appears to be increasing, even after more than 100 days of operation.

Figure 10b suggests (but does not by itself prove) that methane production is linked to CM degradation. As soon as DCM additions were stopped, methane continued to accumulate, but at a slower rate. The pathway for CM degradation is not yet known. One possibility is that CM serves as an electron acceptor, in which case reductive dechlorination would be the principal transformation mechanism. Reductive dechlorination of CM would require an external electron donor and result in formation of 1.0 mol of methane per mol of CM degraded. Another possibility is that CM is an electron donor, just as DCM is. If so, then 0.75 mol methane would be formed per mol of CM degraded:



The bottles degrading CM have not received any other source of organic compounds for over 82 days (since DCM additions were stopped). Nevertheless, the rate of CM degradation is improving rather than declining (Figure 10). This tends to support the hypothesis that CM serves as an electron donor. Some other electron donor may have been transferred with the culture from the 2-L reactor; however, after over 100 days of operation, it seems reasonable to assume that none remains. In bottles receiving no DCM or CM, methane output does taper off, albeit slowly, before 100 days; this indirectly indicates exhaustion of any external electron donors in the bottle. Furthermore, addition of methanol did not stimulate the rate of CM degradation; stimulation of reductive dechlorination with methanol has been demonstrated with other halogenated aliphatics [2].

The ratio of methane output to CM degradation thus far does not clearly support either hypothesis. In the bottle depicted in Figure 10, 0.66 mol of methane was formed per mol of CM degraded over the last 20 days of operation, versus 1.1 mol of methane formed per mol of CM degraded in the duplicate bottle. These ratios are subject to considerable error because the amounts of CM and methane being measured over long periods is relatively low. If the rate of CM degradation continues to improve, more accurate measurement of the stoichiometry will be possible, since larger quantities will be involved.

The results of this work conclusively demonstrate that CM can be biodegraded under methanogenic conditions. Previous studies have noted appearance and subsequent disappearance of CM when degrading DCM and CF under methanogenic conditions [3, 5]. However, the possible role of losses and abiotic reactions were not explored. Also, the peak level of CM observed in those studies never exceeded 0.6  $\mu\text{mol}$  per bottle (the same type of serum bottle, with the same volume of culture used in this study). Previous work by Hartmans et al. demonstrated the biodegradability of CM under aerobic conditions [6].

#### **4. Biodegradation of Bromomethane**

Results obtained thus far with BM are very preliminary because a suitable type of septum has not yet been identified for retaining this compound. Grey-butyl rubber septa are acceptable for use with DCM, DBM, BCM, and CM, but not for BM. In water controls, 94% of the BM is lost from the serum bottles in 25 days. This is an unacceptably high rate.

Before the high rate of BM loss through the septa was known, six inoculated bottles were set-up using the same protocol as described for the other compounds. Some useful results were obtained, despite excessive loss of BM:

1) BM inhibited methane production, though not as completely as DBM and BCM; inhibition was observed in bottles receiving 10  $\mu\text{mol}$  BM as well as those receiving as little as 2  $\mu\text{mol}$ .

2) In duplicate bottles receiving both BM (2  $\mu\text{mol}$ ) and DCM (10  $\mu\text{mol}$ ), DCM degradation was completely stopped.

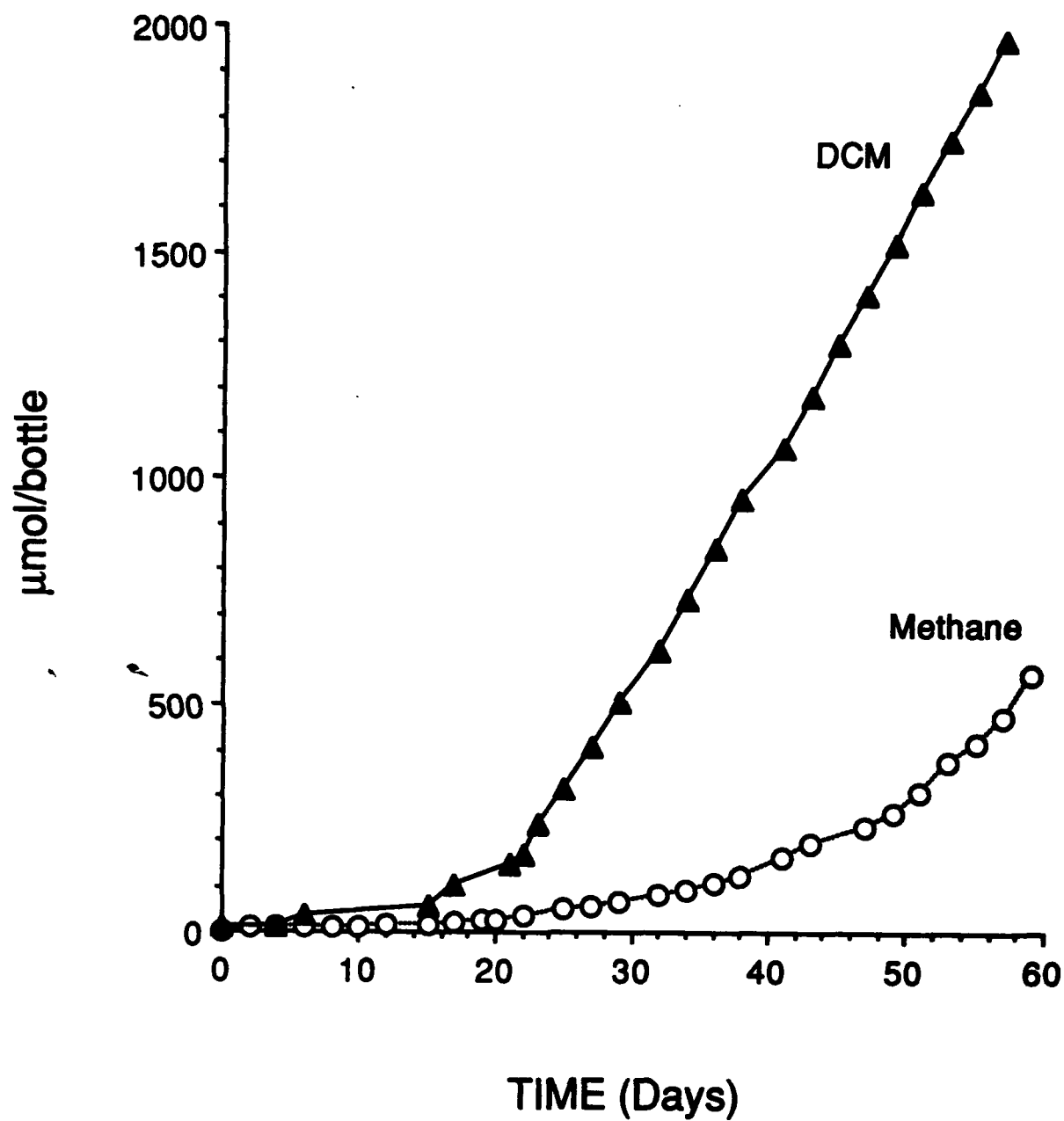
3) CM accumulated in inoculated as well as autoclaved-inoculated bottles. The apparent method of CM formation was abiotic substitution of  $\text{Cl}^-$  for  $\text{Br}^-$ . A high concentration of  $\text{Cl}^-$  in the basal medium (approximately 37 mM) favors the energetics of this reaction.

Numerous septa are now being tested for their ability to retain BM. Once one is found, these experiments will be repeated so that the contribution of biodegradation to disappearance of BM can be measured. Nevertheless, the results summarized above are not expected to change.

The experiments conducted with DBM, FCM, and BM demonstrated that brominated methanes are far more difficult to metabolize than DCM, at least for the DCM-degrading enrichment culture employed. This obviously makes brominated compounds a poor choice as a substrate for serial dilution of DCM-degrading organisms. The DCM-degrading enrichment culture does have the capability to degrade CM, perhaps using a pathway similar to that involved in DCM degradation. At this point, however, the rate of CM metabolism is much slower compared to DCM. In addition to CM and DCM, the enrichment culture appears to be versatile enough to metabolize other chlorinated methanes, described below.

### C. Studies with Chloroform

The ability of DCM-degrading enrichment cultures to metabolize CF was examined in an experiment involving 10 bottles. Each bottle received 100 mL from the 2-L "mother" culture. Two served as DCM-only controls; they received an initial dose of 16.4  $\mu\text{mol}$  of DCM and degraded it within two days. The amount added and degraded every other day was gradually increased to 113  $\mu\text{mol}$  of DCM by day 33, then held at that level for the duration of the experiment. Methane was consistently produced in these bottles, but below the amount predicted based on stoichiometric conversion of DCM to methane (Figure 11). This observation is consistent with



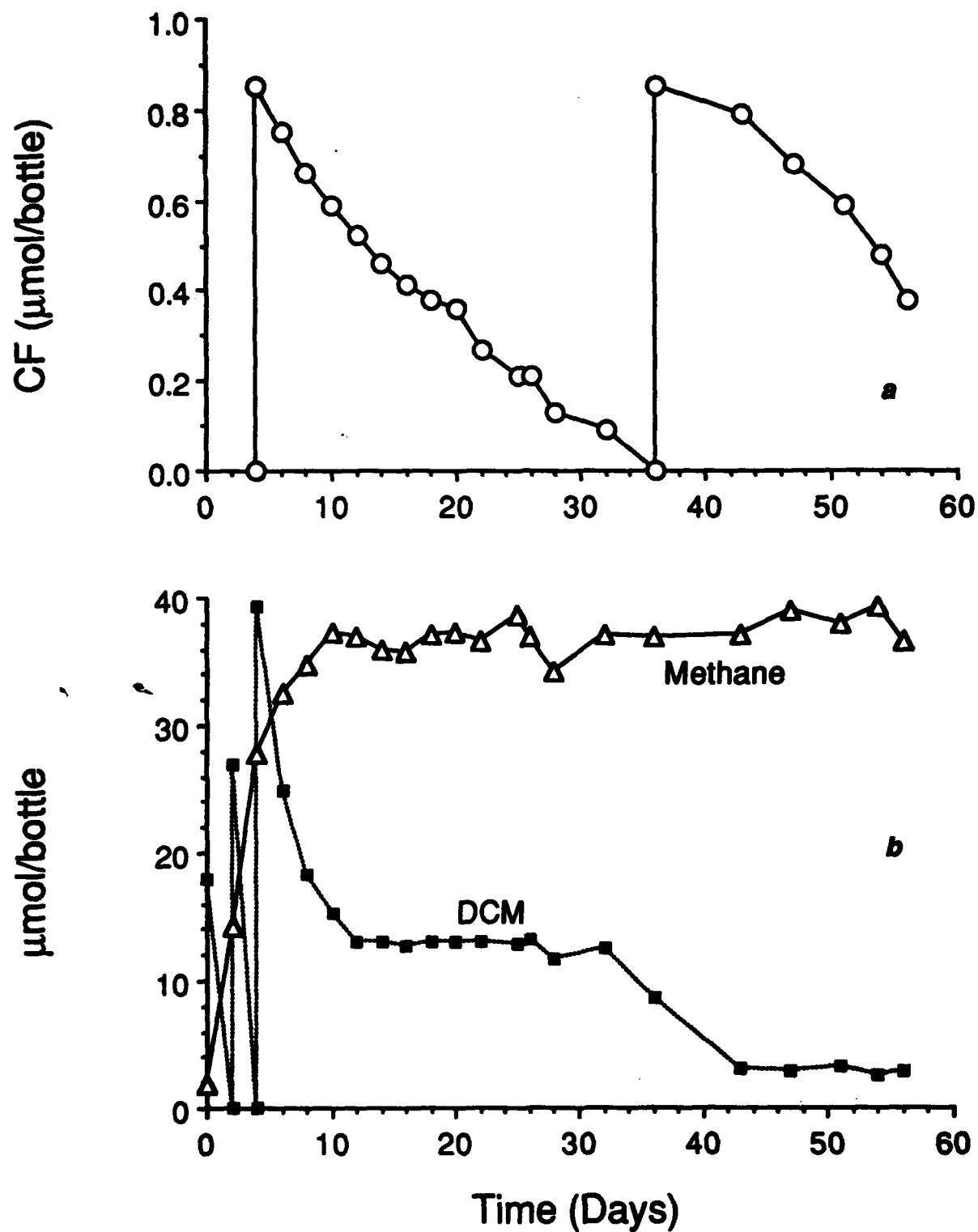
**Figure 11:** Cumulative DCM degradation and methane formation in a typical control bottle receiving only DCM. The highest amount of DCM repetitively added was 113  $\mu\text{mol}$ .

previous findings that levels of DCM above approximately 15  $\mu\text{mol}$  per bottle inhibit methanogens, particularly acetoclastic methanogens [3].

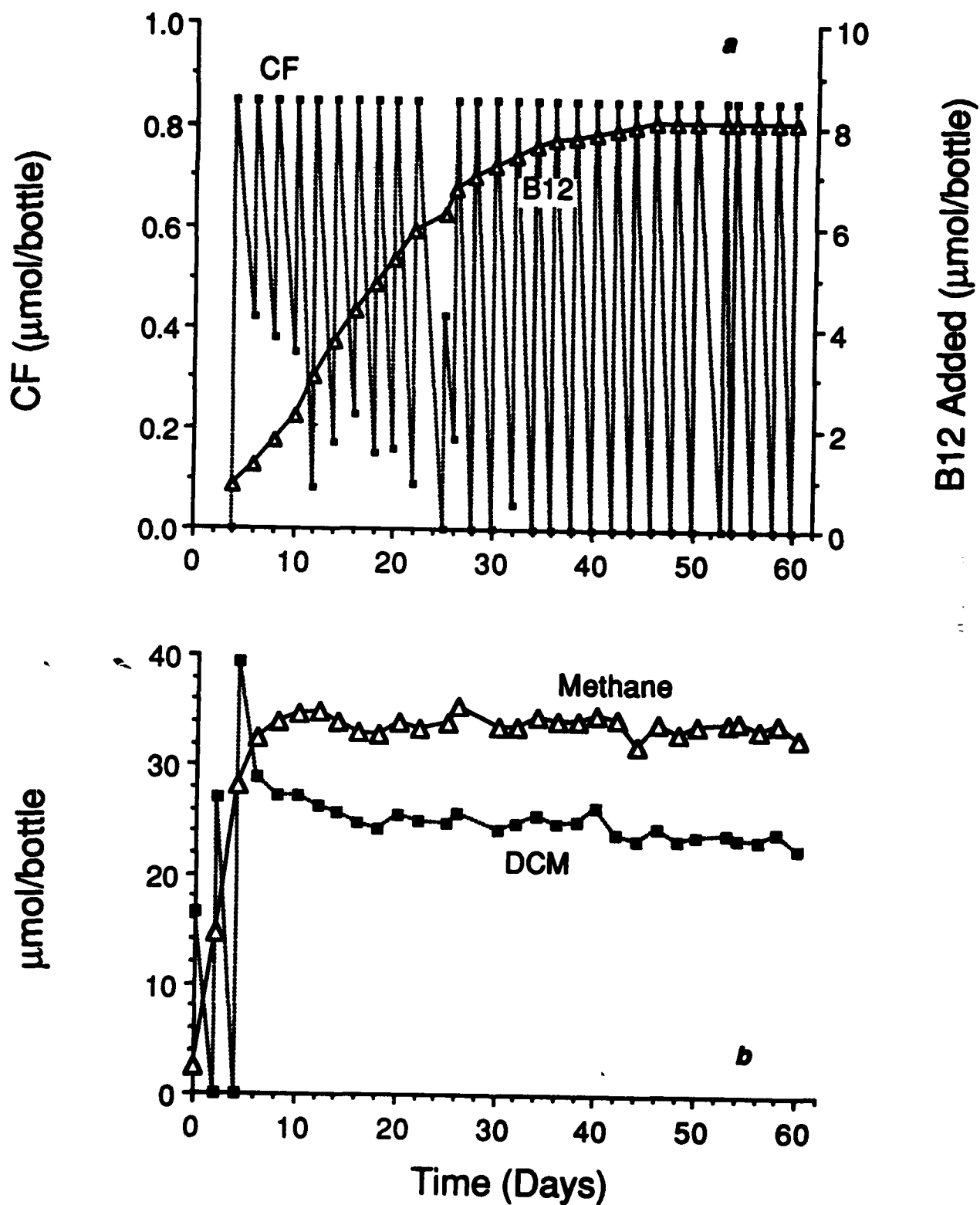
Two bottles were also started on DCM; along with the third DCM addition on day 4, 0.85  $\mu\text{mol}$  of CF was also provided (Figure 12). After 32 days, the CF was degraded below its detection limit. The rate of DCM degradation was sharply curtailed in the presence of CF, and methane production essentially stopped by day 10. A second 0.85  $\mu\text{mol}$  addition of CF degraded at a slightly faster rate, and at the same time DCM degradation improved. It remains to be seen whether or not CF degradation can be sustained and even accelerated.

Two bottles were treated identically to the ones described above except that whenever CF was added, vitamin B<sub>12</sub> (cobalamin) was also added. The first dose of B<sub>12</sub> was 0.85  $\mu\text{mol}$ , the same amount as CF on a molar basis. Subsequent B<sub>12</sub> additions were gradually reduced so that, by day 48, additions were stopped. As shown in Figure 13, the presence of B<sub>12</sub> dramatically improved the rate of CF degradation compared to the bottles not receiving B<sub>12</sub>. Repetitive CF degradation has sustained for more than 50 days, even after B<sub>12</sub> additions were stopped. This suggests a catalytic role for B<sub>12</sub>, in contrast to the possibility that the B<sub>12</sub> was merely bonding to CF and not transforming it. At least to this point of operation, there appears to be no need for an auxiliary electron donor to sustain CF degradation. Work is now underway to gradually increase the amount of CF added. In the event that the rate of degradation slows, more B<sub>12</sub> will be added.

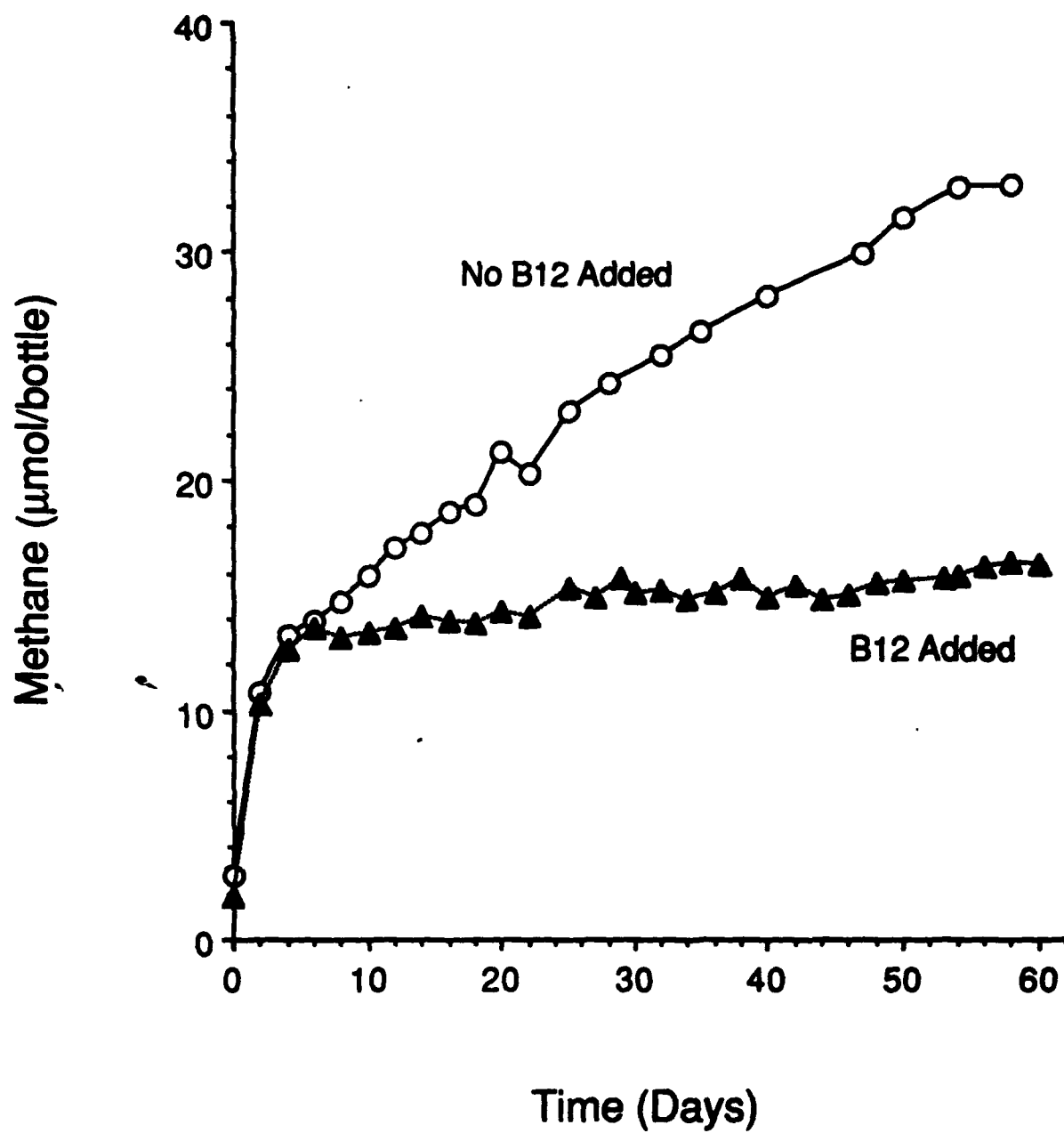
One conceivable way B<sub>12</sub> could have facilitated CF degradation was by merely serving as an electron donor to sustain reductive dechlorination. Four bottles were set-up to examine this possibility, using total methane output as a surrogate measure of potential electron donor availability. As shown in Figure 14, addition of B<sub>12</sub> (at exactly the same rate as the bottles depicted in Figure 13) actually inhibited methane output, yielding progressively less than the bottles receiving nothing. This suggests that B<sub>12</sub> did not serve as an electron donor for reductive dechlorination; if anything, the presence of B<sub>12</sub> reduced the pool of available electron donors by completely inhibiting methanogenesis.



**Figure 12:** CF biodegradation in the absence of added  $B_{12}$  (a); CF inhibited DCM degradation and methanogenesis (b).



**Figure 13:** CF biodegradation and cumulative B<sub>12</sub> added (a), and the effect of CF on DCM degradation and methane production (b).



**Figure 14:** Methane production in bottles with and without addition of B<sub>12</sub>; no other compounds were added.



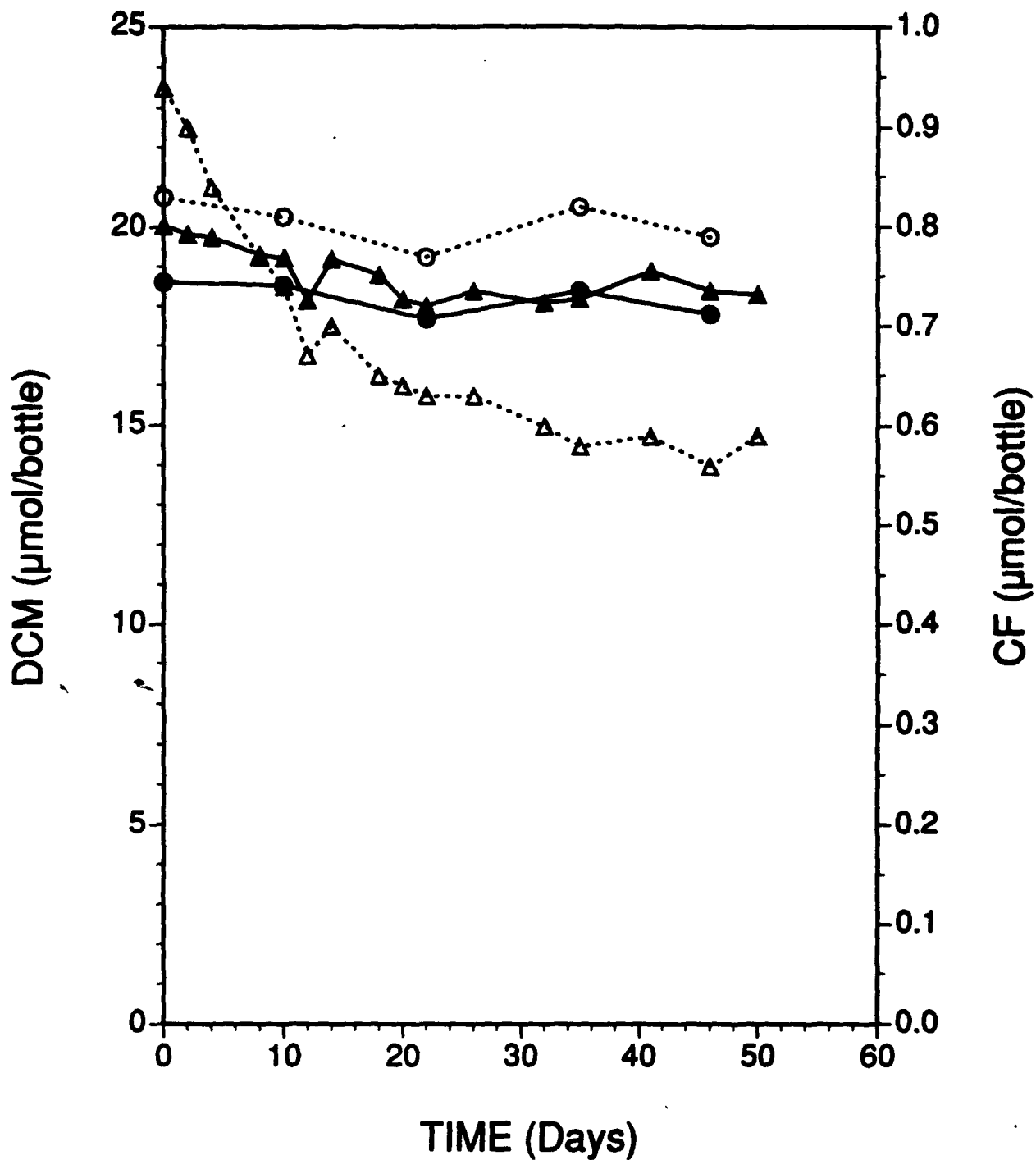
Loss of DCM from autoclaved-inoculated and water controls was minimal, as expected. The percentage loss of CF was also relatively low, though somewhat higher in the autoclaved controls than in the water controls (Figure 15). These data confirm that the disappearance of CF in the inoculated bottles was a consequence of biological activity.

[ $^{14}\text{C}$ ]CF was used to examine the fate of CF when biodegraded in the presence of  $\text{B}_{12}$ . Four bottles were started with culture from the 2-L reactor. This time, however, DCM was added only once and allowed to degrade completely (in two days) before adding  $0.85\ \mu\text{mol}$  of CF. Two of the bottles received  $\text{B}_{12}$  along with CF, two did not. In the two bottles receiving  $\text{B}_{12}$ , the amount added with each addition of CF was gradually decreased (Figure 16b). By day 29, no further additions of  $\text{B}_{12}$  were made. Although trace amounts of DCM were noted occasionally, DCM did not accumulate as an intermediate, and none was evident past day 20. CF additions inhibited methanogenesis almost completely.

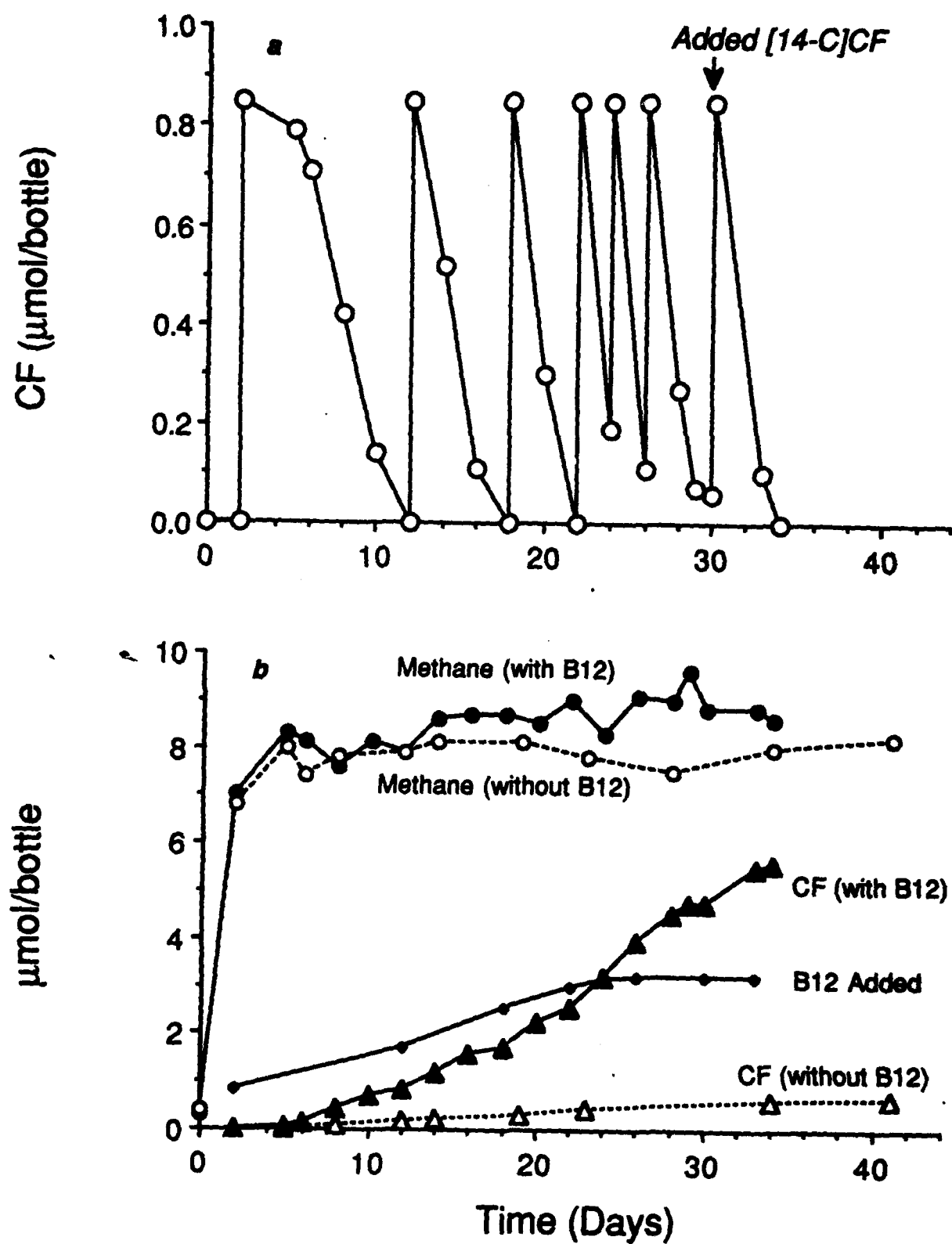
In the two bottles that did not receive  $\text{B}_{12}$ , the rate of CF degradation was much slower (Figure 16b). Between day 2 and day 41, only  $0.66\ \mu\text{mol}$  of CF was degraded, versus  $5.59\ \mu\text{mol}$  of CF by day 34 in bottles with  $\text{B}_{12}$  added. Furthermore, DCM accumulated to a level of  $0.22\ \mu\text{mol}$  by day 41 in the bottles not receiving  $\text{B}_{12}$ . Methanogenesis was inhibited in all four bottles by the continuing presence of CF.

In the two bottles receiving  $\text{B}_{12}$ , the final  $0.85\ \mu\text{mol}$  addition of CF on day 30 was accompanied by approximately  $2.7 \times 10^6$  dpm of [ $^{14}\text{C}$ ]CF (Figure 16a). Routine headspace analysis on day 34 indicated that the CF level was below the detection limit, so the bottles were sacrificed to determine the distribution of  $^{14}\text{C}$ . The methods used to assay  $^{14}\text{C}$  products, both volatile and nonvolatile, were the same as those previously described [3].

Results of the [ $^{14}\text{C}$ ]CF experiment are presented in Table 2. Slightly more than three quarters of the label was recovered as  $^{14}\text{CO}_2$ , indicating that the predominant mechanism of CF metabolism was oxidation. The next most significant product was approximately 10% carbon monoxide (CO). Additional work is needed to confirm that this was indeed CO, but the evidence



**Figure 15:** Loss of DCM from an autoclaved-inoculated control bottle ( $\blacktriangle$ ) and a water control bottle ( $\bullet$ ), and loss of CF from an autoclaved-inoculated control bottle ( $\triangle$ ) and a water control bottle ( $\circ$ ).



**Figure 16:** CF and [14C]CF biodegradation in a bottle supplemented with B<sub>12</sub> (a); the effect of B<sub>12</sub> on CF degradation and the effect of CF on methane production (b).

**Table 2:**  
**Distribution of  $^{14}\text{C}$  from Biodegradation of [ $^{14}\text{C}$ ]CF**

	<u>Bottle #1</u>		<u>Bottle #2</u>	
	dpm	%	dpm	%
$\text{CO}_2^a$	1,981,217	78.1	1,921,332	76.1
$\text{CO}^b$	250,080	9.9	268,320	10.6
$\text{CH}_4$	-		-	
Soluble Nonstrippable Residue	163,161	6.4	205,093	8.1
Nonsoluble Nonstrippable Residue	68,703	2.7	31,656	1.3
CF	68,942	2.7	81,564	3.2
DCM	5,652	0.22	15,822	0.63
CM	<u>-</u>		<u>-</u>	
Total $^{14}\text{C}$ Recovered <sup>c</sup>	2,537,755		2,523,787	

<sup>a</sup> Confirmed by precipitation with  $\text{Ba}(\text{OH})_2$ .

<sup>b</sup> Based on  $^{14}\text{CO}$  in the headspace only; the amount dissolved in the liquid was not included, since the value of Henry's Constant for  $\text{CO}$  at  $35^\circ\text{C}$  has not yet been measured.

<sup>c</sup> The total amount of  $^{14}\text{C}$  added was 2,694,000 dpm; therefore the recovery of  $^{14}\text{C}$  was 94.2% in Bottle #1, 93.7% in Bottle #2.

collected thus far is reasonably convincing. This portion of the  $^{14}\text{C}$  was collected from a headspace sample injected onto a Carbosieve SII column, which was then routed through a combustion tube prior to trapping in NaOH. The retention time of CO on the Carbosieve SII (at  $150^\circ\text{C}$  isothermal, He carrier flow at 30 mL/min) was tested and occurred well before methane, with no overlap. The  $^{14}\text{C}$  that was collected corresponded to the retention time of CO, whereas none of the  $^{14}\text{C}$  was collected during the methane fraction. The only other possibility considered thus far was  $^{14}\text{C}$ -methanethiol, but this was also ruled out because no  $^{14}\text{C}$  was collected during the methanethiol fraction eluting from the 1% SP-1000 Carbopack B column.

The next highest fraction was  $^{14}\text{C}$ -soluble nonstrippable residue, comprising approximately 75% of the  $^{14}\text{C}$  recovered. Work is currently underway to determine the distribution of  $^{14}\text{C}$  within this category, using an HPLC system with a BIORAD HPX-87H ion exchange column to separate the various fractions. Using a mobile phase (0.013 N  $\text{H}_2\text{SO}_4$ ) flow rate of 0.7 mL/min and a column temperature of  $30^\circ\text{C}$ , preliminary results indicate the following distribution: 33-41% elutes just prior to acetate; 12-15% elutes in the acetate fraction; and 37-49% elutes in an interval extending from 32 to 40 minutes, long after butyrate elutes. The fraction eluting before acetate may be formate or formaldehyde; the 32-40 minute fraction may be a form of CF bound to  $\text{B}_{12}$ .

About 2% of the label was recovered as  $^{14}\text{C}$ -nonsoluble nonstrippable residue. This may be  $^{14}\text{C}$  sorbed to particulate matter (i.e.,  $> 0.45\ \mu\text{m}$ ) or biomass. Considerably more work will be needed to ascertain if CF actually does serve as a growth substrate. The other  $^{14}\text{C}$ -labeled compounds recovered were approximately 3% CF and 0.4% DCM. The sum of the dpm identified as  $\text{CO}_2 + \text{CO} + \text{nonstrippable residue} + \text{CF} + \text{DCM}$  amounted to an average of 94% of the total  $^{14}\text{C}$  added to the bottles. The purity of the [ $^{14}\text{C}$ ]CF stock was checked by adding the same amount of activity to a water control as was added to the inoculated bottles. Approximately 99% of the  $^{14}\text{C}$  recovered from this bottle was [ $^{14}\text{C}$ ]CF, with only traces of [ $^{14}\text{C}$ ]DCM and  $^{14}\text{CO}_2$ .

Additional experiments with [ $^{14}\text{C}$ ]CF will be conducted to provide a more complete picture of the metabolic pathway. The high amount of  $^{14}\text{CO}_2$  formed is in agreement with previous work

by Gossett [5]. Of particular interest from this study was the recovery of a significant amount of CO. This suggests that carbon monoxide dehydrogenase plays a role in oxidation of CF to CO<sub>2</sub>; it also tends to support involvement of the acetyl-CoA pathway in metabolism of CF.

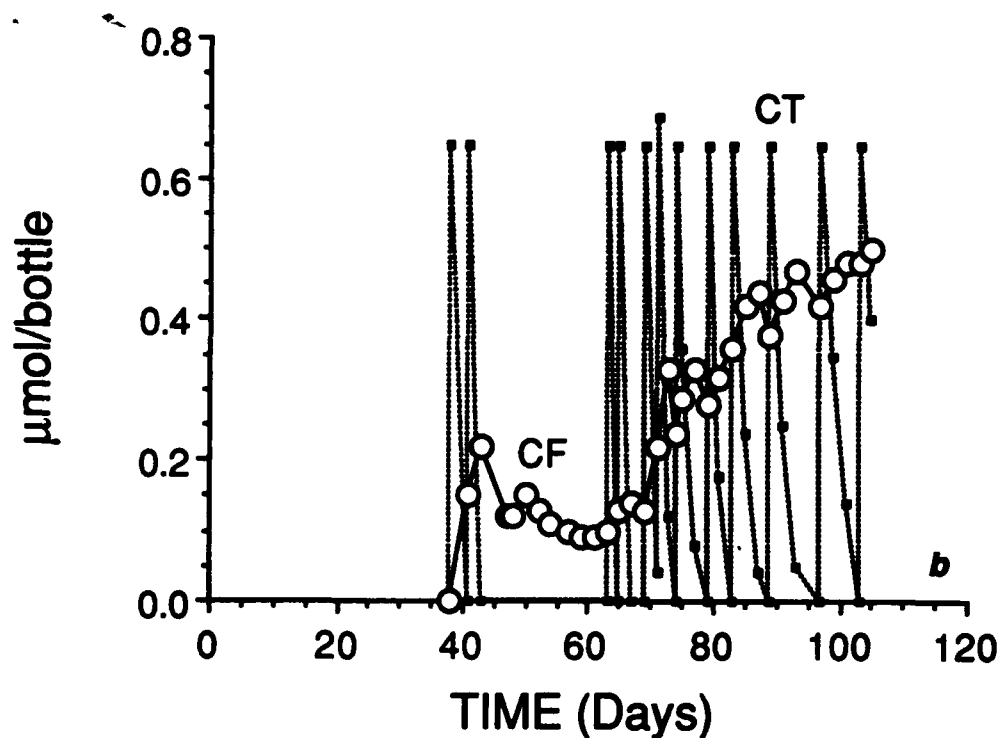
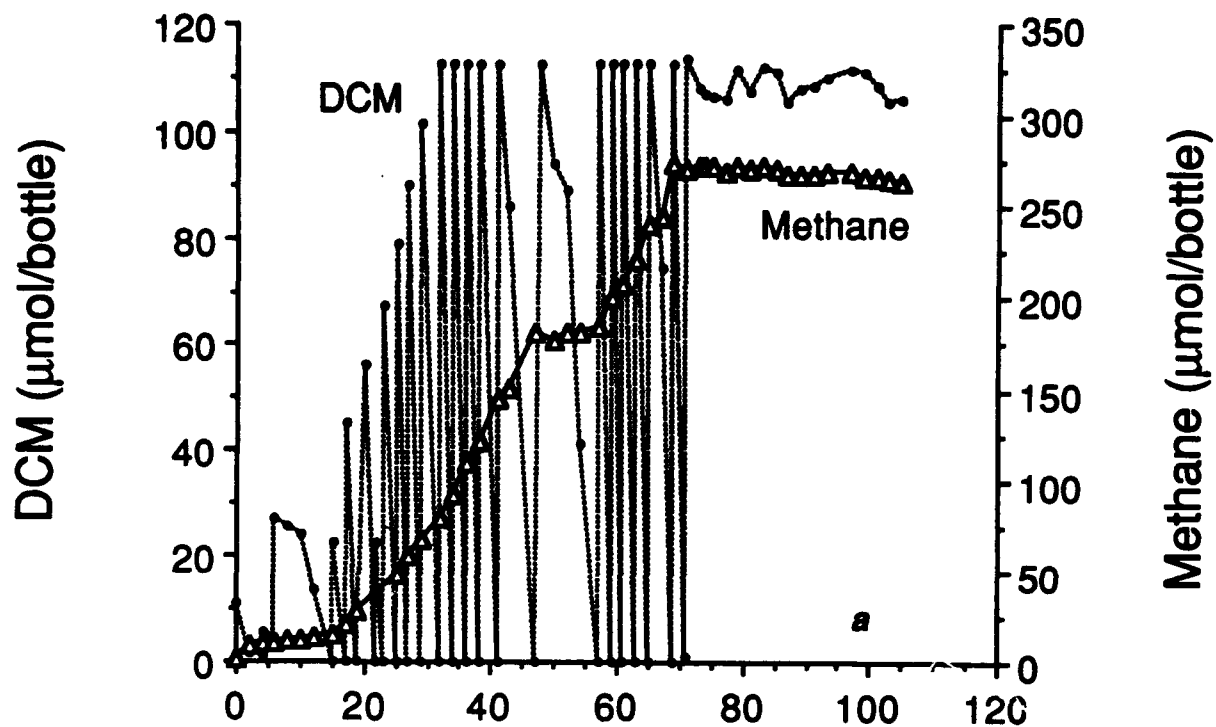
#### **D. Studies with Carbon Tetrachloride**

Although less work has been accomplished thus far with CT than with CF, some preliminary data are available. Two bottles were set-up from the 2-L mother culture and DCM additions were gradually increased from 11 to 113  $\mu$ mol per bottle every other day (Figure 17). Shortly thereafter, 0.65  $\mu$ mol CT was added on day 38; this disappeared by day 41, and another dose of CT was added; it too was rapidly degraded. As CF began to accumulate, the rate of DCM degradation started to slow down and methane output stopped completely. CT additions were stopped on day 43, resulting in a decline in CF and an eventual improvement in the rate of DCM degradation and methane output by day 57. Repetitive additions of CT resumed on day 63, and continued through day 105. Over this interval, CF once again accumulated, and DCM degradation and methane production stopped completely. Most significantly, however, is the fact that CT degradation was sustained for so long without the addition of an electron donor other than DCM. With time, the rate of CT degradation slowed, leaving open the question of whether or not an electron donor is required.

Experiments with B<sub>12</sub> and CT will be conducted just as they were with CF, including the use of [<sup>14</sup>C]CT to ascertain the distribution of transformation products. Recent work by Gantzer and Wackett [4] demonstrating the effectiveness of B<sub>12</sub> for reductively dechlorinating CT abiotically suggests that this approach should work with the DCM-degrading enrichment culture.

#### **E. References**

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**Figure 17:** CT biodegradation in a DCM-degrading enrichment culture.

2. **Freedman, D. L. and J. M. Gossett.** 1989. Biological reductive dechlorination of tetrachloroethylene and trichloroethylene to ethylene under methanogenic conditions. *Appl. Environ. Microbiol.* **55**(9): 2144-2151.
3. **Freedman, D. L. and J. M. Gossett.** 1991. Biodegradation of dichloromethane and its utilization as a growth substrate under methanogenic conditions. To be published in *Appl. Environ. Microbiol.* **57**(10).
4. **Gantzer, C. J. and L. P. Wackett.** 1991. Reductive dechlorination catalyzed by bacterial transition-metal coenzymes. *Environ. Sci. Technol.* **25**(4): 715-722.
5. **Gossett, J. M.** 1985. Anaerobic degradation of C<sub>1</sub> and C<sub>2</sub> chlorinated hydrocarbons. NTIS no. AD-A165 005/0. Engineering & Services Laboratory, U. S. Air Force Engineering and Services Center: Tyndall AFB, FL.
6. **Hartmans, S., A. Schmucke, A. M. Cook and T. Leisinger.** 1986. Methyl chloride: naturally occurring toxicant and C-1 growth substrate. *J. General Microbiol.* **132**: 1139-1142.

### III: ARTICLES

Thus far, one paper stemming from this research has been accepted for publication; a copy is contained in Appendix B:

Freedman, D. L. and J. M. Gossett. 1991. Biodegradation of dichloromethane in a fixed-film reactor under methanogenic conditions. To be published in the Symposium Proceedings, *"In Situ and On-Site Bioreclamation,"* Butterworth, Inc.

Several articles are planned for publication and will most likely be submitted to *Applied and Environmental Microbiology*. Possible titles are:

- ° Biodegradability of dibromomethane, bromochloromethane, chloromethane and bromomethane under methanogenic conditions.
- ° Effect of temperature on the biodegradability of dichloromethane under methanogenic conditions.
- ° Biodegradation of chloroform under methanogenic conditions.
- ° Isolation and characterization of a dichloromethane-degrading acetogen.



#### **IV: PROFESSIONAL PERSONNEL**

This project began as a post-doctoral fellowship awarded to **Dr. Freedman** while he was at Cornell University. At that time, **Dr. James M. Gossett** (School of Civil and Environmental Engineering) was his post-doctoral advisor; a considerable amount of guidance was also provided by **Dr. Stephen Zinder** (Department of Microbiology). Six months into the project, **Dr. Freedman** took a position at the University of Illinois. The Air Force Office of Scientific Research graciously permitted him to transfer the project from Cornell to the University of Illinois. After a six month delay, the project was restarted. Two graduate students — **Jennifer Becker** and **Gregory Delaney** — are currently supported by the project. Both are candidates for the Master of Science degree in Environmental Engineering, and both are expected to complete their thesis by January, 1992.

**Ms. Becker** has been doing the research on CF and CT. A likely title for her thesis is "Biotransformation Pathways for Chloroform under Methanogenic Conditions." The work she started on CT will be picked up by a doctoral candidate, **Syed Hashsham**. **Mr. Hashsham** will be starting this fall; in addition to working with CT, he will work on isolation of the DCM-degrading organisms.

**Mr. Delaney** has been doing the research on DBM, BCM, CM, and BM. A likely title for his thesis is "Biodegradation of Dibromomethane, Bromochloromethane, Chloromethane, and Bromomethane under Methanogenic Conditions." **Mr. Delaney** is also working on the effects of temperature on the rate of DCM degradation, to determine if DCM metabolism can be sustained at temperatures below 20°C.

## **V: INTERACTIONS (COUPLING ACTIVITIES)**

Results from this research have been presented orally at the International Symposium, *"In Situ and On-Site Bioreclamation,"* sponsored by Battelle, Inc. The symposium was held in San Diego, California, March 19-21, 1991. A large number of government personnel were present, including representatives from the Air Force Engineering and Services Center.

Dr. Freedman is currently working with Rick Scholze at the U. S. Army Construction Engineering Research Lab, Champaign, Illinois on applications of this research to remediation of halogenated solvent contamination of Army installations.

## **VI: DISCOVERIES, INVENTIONS, PATENTS & APPLICATIONS**

No new inventions or patent disclosures have resulted from this research. However, several of the findings have direct relevance to remediation of water contaminated with polychlorinated methanes. Perhaps most significantly, the principal metabolic products of chloroform degradation by a DCM-degrading enrichment culture have been identified as CO<sub>2</sub>, CO, and acetic acid, all of which are environmentally acceptable. The key to sustaining chloroform degradation has been use of vitamin B<sub>12</sub>, in catalytic quantities. Results thus far indicate that chloroform degradation under methanogenic conditions does require the presence of an external electron donor. This research has also shown that brominated methanes are much more difficult to biodegrade than chlorinated methanes under methanogenic conditions.

*Title:*

**Biodegradation of Dichloromethane and its  
Utilization as a Growth Substrate Under  
Methanogenic Conditions**

*Running Title:*

**Anaerobic Biodegradation of Dichloromethane**

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### ***Abstract***

Biodegradation of dichloromethane (DCM) to environmentally acceptable products was demonstrated under methanogenic conditions (35°C). When DCM was supplied to enrichment cultures as the sole organic compound, at a low enough concentration to avoid inhibition of methanogenesis, the molar ratio of CH<sub>4</sub> formed to DCM consumed (0.473) was very close to the amount predicted by stoichiometric conservation of electrons. DCM degradation was also demonstrated when methanogenesis was partially inhibited (with 0.5-1.5 mM 2-bromoethanesulfonate or approximately 2 mM DCM) or completely stopped (with 50-55.5 mM 2-bromoethanesulfonate). Addition of a eubacterial inhibitor (vancomycin, 100 mg/liter) greatly reduced the rate of DCM degradation. <sup>14</sup>CO<sub>2</sub> was the principal product of [<sup>14</sup>C]DCM degradation, followed by <sup>14</sup>CH<sub>4</sub> (when methanogenesis was uninhibited) or <sup>14</sup>CH<sub>3</sub>COOH (when methanogenesis was partially or completely inhibited). Hydrogen accumulated during DCM degradation, then returned to background levels when DCM was consumed. These results suggested that non-methanogenic organisms mediated DCM degradation, oxidizing a portion to CO<sub>2</sub> and fermenting the remainder to acetate; acetate formation suggested involvement of an acetogen. Methanogens in the enrichment culture then converted the products of DCM degradation to CH<sub>4</sub>. Aceticlastic methanogens were more easily inhibited by 2-bromoethanesulfonate and DCM than CO<sub>2</sub>-reducing methanogens. When DCM was the sole organic-carbon and electron-donor source supplied, its use as a growth substrate was demonstrated. The highest observed yield was 0.085 grams of suspended organic carbon formed per gram of DCM carbon consumed. Approximately 85% of the biomass formed was attributable to the growth of nonmethanogens, 15% to methanogens.

## INTRODUCTION

Dichloromethane (DCM) has been in widespread use for several decades. Since 1970, annual U. S. production has ranged from 212 to 287 million kilograms, with the principal application being paint removal (34). DCM is one of 14 volatile organic compounds regulated under the Safe Drinking Water Act Amendments of 1986; it has been shown to cause lung and liver cancer in mice (25). Because of its relatively low Henry's constant (14), DCM is difficult to remove from contaminated groundwater by air stripping, one of the most commonly applied remediation techniques for volatile organics (22).

Surprisingly little information is available concerning the behavior of DCM under methanogenic conditions. DCM is a potent inhibitor of methanogenesis (28, 32, 35). However, inhibition typically diminishes as cultures acclimate to continuous DCM addition. Stuckey et al. (28) observed the ability of a mixed culture to acclimate even when the digester concentration of DCM reached 10 mg/liter. Biological degradation of DCM has been demonstrated conclusively by Gossett (13), using mixed batch cultures which repetitively consumed 8 mg/liter additions of DCM. The principal biotransformation products of [ $^{14}\text{C}$ ]DCM were  $^{14}\text{CO}_2$  (approximately 73%) and a nonstrippable residue (21%), about one-half of which was soluble. Because the identity of the nonstrippable residue was not determined, it was impossible to ascertain whether or not this major product was environmentally acceptable. Evidence for biodegradation of DCM has also come from studies with continuous-flow reactors (2, 28). However, these studies did not investigate the products of DCM transformation or the organisms responsible for carrying them out. Formation of DCM from biodegradation of carbon tetrachloride (7) and chloroform (6, 13) has also been shown; both compounds inhibit further degradation of the DCM formed.

In this study, anaerobic enrichment cultures and specific inhibitors were used to investigate the roles of methanogens and nonmethanogens in mediating DCM degradation. When DCM was supplied as the sole organic-carbon and electron-donor source, essentially stoichiometric amounts of methane were produced from the DCM consumed. The use of DCM as a growth substrate was also demonstrated. Volatile and nonvolatile products of DCM biotransformation were identified

using [ $^{14}\text{C}$ ]DCM and measurements of hydrogen formation. Based on the cumulative evidence, a model for DCM degradation under methanogenic conditions is proposed.

## MATERIALS AND METHODS

**Chemicals.** DCM was obtained in neat liquid form (99 mol % pure; Fisher Scientific); it was added to cultures from saturated-water stock solutions (approximately 240 mM DCM). Chloromethane was purchased dissolved in methanol (200 mg/liter, 1-ml ampoule; Supelco, Inc.). Methane and ethylene were obtained in gaseous form (99+%; Scott Specialty Gases). 2-Bromoethanesulfonic acid (sodium salt, 98%) was purchased from Aldrich Chemical Co. Vancomycin hydrochloride (963  $\mu\text{g}/\text{mg}$ ) was obtained from Sigma Chemical Co. (St. Louis, MO) and dissolved in water (10.4 g/liter). [ $^{14}\text{C}$ ]DCM (Sigma Radiochemical) was diluted in 150 ml distilled deionized water and stored in a 160-ml serum bottle, capped with a grey-butyl rubber septum (Wheaton) and aluminum crimp cap. The [ $^{14}\text{C}$ ]DCM stock solution contained  $2.80 \times 10^7$  dpm/ml (4.62 mM DCM); gas chromatographic (GC) analysis of the stock bottle headspace indicated the presence of an unidentified contaminant, which was not radiolabeled. There was no indication that this contaminant interfered (e.g., as an inhibitor or electron donor) with the DCM degradation studies.  $^{14}\text{CH}_3\text{COOH}$  and  $\text{CH}_3^{14}\text{COOH}$  (Sigma Radiochemical) were diluted in distilled deionized water. To ensure purity, aliquots of each were processed by HPLC (described below), and the fraction corresponding to acetic acid (HAc) was added to 150 ml of 0.013 N  $\text{H}_2\text{SO}_4$ . The final  $^{14}\text{CH}_3\text{COOH}$  stock contained 1710 dpm/ml; the final  $\text{CH}_3^{14}\text{COOH}$  stock contained 1850 dpm/ml. ScintiVerse-E<sup>TM</sup> liquid scintillation cocktail was employed (Fisher Scientific).

**Cultures and enrichment procedures.** All experiments were conducted in 160-ml serum bottles containing 100 ml of liquid. The bottles were sealed with slotted grey-butyl rubber septa and aluminum crimp caps. Incubation was conducted at 35°C, with the liquid in contact with the septum (to minimize loss of volatiles), and under quiescent conditions, except where noted. Degradation of DCM was initially achieved in "first-generation" cultures, prepared by anaerobically

transferring 100-ml mixed-liquor samples directly from a laboratory reactor to serum bottles. The laboratory reactor was a 15-liter, stirred, semi-continuous, anaerobic digester operated at 35°C, a residence time of 20 days, and fed a complex substrate (Ensure™), as previously described (10).

In this study, we report the total mass of each compound present in a serum bottle. Most of the DCM resided in the liquid, most of the CH<sub>4</sub> in the headspace: Based on Henry's constants  $[(\text{mol}\cdot\text{m}^{-3} \text{ gas concentration})/(\text{mol}\cdot\text{m}^{-3} \text{ aqueous concentration})]$  of 0.128 (14) and 33.1 (10) for DCM and CH<sub>4</sub>, respectively, at 35°C, 93% of the DCM and 4.8% of the CH<sub>4</sub> was present in the 100 ml of liquid, with the balance in the 60-ml headspace.

A typical initial DCM dose was 8-12 µmol per bottle, or an aqueous concentration of 6.3 to 9.5 mg DCM/liter. Whenever DCM dropped near or below its detection limit, more DCM-saturated stock solution was added. After operating in this mode for at least 80 days, first-generation cultures were used to inoculate second-generation cultures, which were then used to inoculate third-generation cultures, and so on. A 2-10% (v/v) inoculum was employed with each transfer, the balance consisting of basal medium (plus 50 mg/liter HAc, where noted) that was anaerobically prepared and dispensed as previously described (10). It included bicarbonate as pH buffer and 50 mg/liter yeast extract.

Each set of experiments with inoculated bottles was accompanied by duplicate water controls (100 ml distilled deionized water plus DCM) and duplicate inoculated bottles which were autoclaved (121°C, 30 minutes) and cooled before addition of the DCM.

Eighteen bottles (all third- and fourth-generation) were used to examine the fate of [<sup>14</sup>C]DCM. Prior to receipt of [<sup>14</sup>C]DCM, they were subjected to differing conditions. In B-1 through B-8, methanogenesis was uninhibited and DCM (10-12 µmol per bottle) was added repetitively; 10 mg HAc was added along with each DCM dose in bottles B-1 and B-2, 1 mg HAc in B-3 and B-4, 0.1 mg HAc in B-5 and B-6, and no HAc in B-7 and B-8. In B-9 through B-18 no HAc was added and methanogenesis was inhibited (either partially or completely) by adding BES, high doses of DCM, or both. In B-9 and B-10, DCM additions as high as 240 µmol per bottle were made, with no BES added; in B-11, B-12, and B-13 the DCM dose was 10-12 µmol

per bottle along with 0.5-1.5 mM BES; in B-14, B-15 and B-16 the DCM dose was 10-12  $\mu\text{mol}$  per bottle along with 50-55.5 mM BES; and in B-17 and B-18 the DCM dose was 53-84  $\mu\text{mol}$  per bottle along with 50-55.5 mM BES. Since all 18 bottles were used in various other experiments, they had been actively degrading DCM (for 51 to 284 days) prior to receiving [ $^{14}\text{C}$ ]DCM.

In 14 of the bottles (B-1 through B-8, B-11 through B-16), 100  $\mu\text{l}$  of [ $^{14}\text{C}$ ]DCM stock ( $2.49\text{-}2.94 \times 10^6$  dpm) was added once along with the usual dose of unlabeled DCM (10-12  $\mu\text{mol}$  per bottle every other day). The same amount of [ $^{14}\text{C}$ ]DCM stock was also added to a water-control bottle. Following a two-day incubation, the inoculated bottles and the water controls were sacrificed to determine the distribution of  $^{14}\text{C}$ . Average results for the water-control bottles (11 analyzed) indicated that nearly all of the  $^{14}\text{C}$  stock consisted of  $^{14}\text{CH}_2\text{Cl}_2$  (97.0%), with the balance being  $^{14}\text{CO}_2$  (2.7%), and a trace of  $^{14}\text{C}$ -labeled nonstrippable residue, all soluble (0.3%).

The four bottles (B-9, B-10, B-17 and B-18) that received high DCM doses were handled differently; each received five repetitive additions of 25  $\mu\text{l}$  of [ $^{14}\text{C}$ ]DCM stock ( $0.66\text{-}0.72 \times 10^6$  dpm) every other day, along with the usual doses of unlabeled DCM. Water controls received the same additions of [ $^{14}\text{C}$ ]DCM. Two days after the fifth DCM addition, they were sacrificed for  $^{14}\text{C}$  analysis. The amount of DCM-saturated water needed to provide the dose of unlabeled DCM used in these bottles was high enough (e.g., 0.9 ml for 200  $\mu\text{mol}$  DCM) that it had to be accounted for, in order to keep the liquid volume in the serum bottles at 100 ml. An equivalent volume of mixed culture was therefore withdrawn prior to adding the DCM, and a 0.10 ml aliquot was counted in liquid scintillation cocktail. The final accounting for  $^{14}\text{C}$  in these bottles was adjusted for the dpm removed in the five effluent samples, by assuming that its composition was the same as that of the liquid in the bottle at the time it was sacrificed for complete  $^{14}\text{C}$  analysis.

The growth experiment involved four serum bottles. B-9 and B-10 were started with 90 ml of basal medium (containing 50 mg/liter yeast extract) plus 10 ml of DCM-degrading inoculum; they were incubated in an orbital shaker bath. Repetitive DCM additions were gradually increased from approximately 10 to as high as 240  $\mu\text{mol}$  per bottle; these high levels afforded the greatest



opportunity to observe an accumulation of biomass within a reasonable time frame. Control bottles (BC-9 and BC-10) were prepared and operated identically, but received no DCM.

**Volatile and  $^{14}\text{C}$ -labeled volatile compounds.** Volatile organics ( $\text{CH}_4$ , chloromethane, and DCM) were routinely determined by GC (Perkin-Elmer model 8500) analysis of a 0.5-ml headspace sample, using a flame-ionization detector in conjunction with a 3.2-mm x 2.44-m stainless-steel column packed with 1% SP-1000 on 60/80 Carbowack-B (Supelco, Inc.), as previously described (10). Detection limits (nmol/bottle) were 19.4 for chloromethane, 49.2 for DCM, and 3.52 for  $\text{CH}_4$ . Identification and confirmation of DCM and chloromethane were made by GC-mass spectrometry (MS; Finnigan Model 3500 GC-MS, coupled with a Teknivent Interactive GC-MS Data System). Identification was subsequently confirmed by injection of authentic material into the same GC system routinely used for headspace monitoring (SP-1000/Carbowack-B column). Identification of methane was accomplished by matching the retention time of authentic material with its peak from headspace samples of enrichment cultures, using four GC columns operated under different temperature conditions, as previously described (10).

GC calibration factors were measured to directly relate the total mass of compound present in a serum bottle to the GC peak area obtained from a 0.5 ml headspace injection. Known masses of DCM and chloromethane were added to replicate serum bottles containing 100 ml distilled deionized water, allowed to equilibrate at 35°C, then analyzed (0.5 ml headspace) by GC (10, 14). Standard additions of  $\text{CH}_4$  were effected from a neat gaseous stock. Coefficients of variation ( $100 \times \text{standard deviation/mean}$ ) for the calibration factors were 1.89% for DCM, 1.64% for chloromethane, and 2.28% for  $\text{CH}_4$ .

The headspace monitoring method relied on the volatiles being freely available, i.e., unsorbed, uncomplexed. This was a reasonable assumption, judging from a comparison of water controls to inoculated bottles. Soon after preparation of a new generation of enrichments, the GC peak areas obtained for DCM in inoculated bottles were consistently close to those in the water controls.

Volatile  $^{14}\text{C}$ -labeled compounds (DCM and  $\text{CH}_4$ ) were analyzed with a GC-combustion technique, as previously described (10). A 0.5-ml headspace sample was injected onto the GC and the well-separated compounds were routed to a catalytic combustion tube, where they were converted to  $\text{CO}_2$ . Each fraction was then trapped in NaOH and added to liquid scintillation cocktail. [ $^{14}\text{C}$ ]DCM was resolved on the SP-1000/Carbopack B column (4.6- to 7.1-minute fraction), while  $^{14}\text{CH}_4$  and  $^{14}\text{CO}_2$  were resolved on the Carbosieve S-II column. Henry's constants for DCM and  $\text{CH}_4$  at  $35^\circ\text{C}$  (given above) were used to relate the dpm measured from a 0.5 ml headspace injection to the total DCM and  $\text{CH}_4$  dpm in a bottle (10).

**$^{14}\text{CO}_2$  and  $^{14}\text{C}$ -labeled nonvolatile compounds.**  $^{14}\text{CO}_2$  and  $^{14}\text{C}$ -labeled nonvolatile compounds were measured as previously described (10) once analysis of the  $^{14}\text{C}$ -labeled volatile compounds was completed. NaOH was added to serum bottles to drive virtually all of the  $\text{CO}_2$  into the aqueous phase. Aliquots were then removed, acidified (with HAc) and purged with  $\text{N}_2$  through an NaOH trap; the  $^{14}\text{C}$  recovered in NaOH corresponded to  $^{14}\text{CO}_2$ , while the  $^{14}\text{C}$  not purged at acid pH corresponded to nonstrippable residue (NSR). When methanogenesis was inhibited, a significant portion of the [ $^{14}\text{C}$ ]DCM was recovered as NSR, most of which was soluble. Composition of the  $^{14}\text{C}$ -labeled soluble NSR was determined by HPLC (Hewlett Packard 1090) (39). 250- $\mu\text{l}$  samples (preparation described below) were pumped (0.7 ml/min, 0.013 N  $\text{H}_2\text{SO}_4$ ) through a 300-mm ion-exchange column (Bio-Rad Laboratories HPX-87H) into an refractive-index detector (Perkin-Elmer LC-25). As fractions eluted, they were collected in 15 ml of liquid scintillation cocktail.

By operating the ion-exchange column at  $30^\circ\text{C}$  and  $65^\circ\text{C}$  we were able to resolve acetate and methanol from several other suspected degradation products, including formate, formaldehyde, propionate, butyrate, isobutyrate, and ethanol (9). Acetate did not coelute with any of the other compounds tested. There was some overlap between methanol, isobutyrate, and propionate at  $30^\circ\text{C}$ , but virtually none at  $65^\circ\text{C}$ . Conversely, there was overlap between methanol and butyrate at  $65^\circ\text{C}$ , but none at  $30^\circ\text{C}$ . The collection intervals were determined by injecting each

compound (100  $\mu$ l at approximately 100 mM), collecting 0.5-minute fractions, then measuring the amount of compound present in each fraction.

Samples were prepared for HPLC analysis by filtering (0.45  $\mu$ m) 20.0 ml of culture that had received NaOH, acidifying with HCl (0.5 ml), purging with N<sub>2</sub> for 30 minutes, then diluting to 25.0 ml. Filtering prior to acidification minimized the possibility of acid-hydrolyzing (and therefore solubilizing) any of the non-filterable material. Also, using HCl for acidification instead of HAc (as used in the <sup>14</sup>C-labeled NSR analysis) made it possible to quantify the total HAc present.

In all of the samples tested the only HPLC fractions that contained significant amounts of radioactivity were those corresponding to HAc and methanol. Confirmation that these fractions actually did contain HAc and methanol was obtained by GC analysis, using a Nukol capillary column (15 m x 0.53 mm ID, 0.5- $\mu$ m film; Supelco) and flame-ionization detector. The column temperature was 80°C for 2 minutes, increased at 20°C to 200°C, then held for 4.5 minutes; the carrier-gas (helium) flow rate was 5 ml/minute. The retention time of the predominant peak in the methanol fraction (1.7 min) matched the retention time of a methanol standard; likewise, the retention time of the predominant peak in the HAc fraction (6.3 min) matched the retention time of an HAc standard.

Additional confirmation of the HAc HPLC fraction was obtained by electron impact GC-MS (Finnigan model 3300). Mass spectra results for the HAc HPLC fraction matched those for an HAc standard (9). The low concentration of methanol in its HPLC fraction prohibited similar direct confirmation by GC-MS. Instead, single-ion monitoring of the MeOH HPLC fraction was employed. The presence of a compound having an m/e value of 31 was confirmed (9). This limited the possible structures to  $\cdot$ OCH<sub>3</sub>,  $\cdot$ OCH<sub>2</sub>O, or CH<sub>3</sub>NH<sub>2</sub>, with the possible origin of the fragment being a primary aliphatic alcohol, methoxy derivatives, methyl esters, dimethyl acetals and ketals, and CH<sub>2</sub>OH branched chains (15). The combined evidence from HPLC, GC, and GC-MS analysis support the conclusion that the compound was methanol.

The efficiency of the HPLC method for recovering the dpm in soluble NSR samples was evaluated as the cumulative dpm recovered from fractions collected off the HPLC (HAc + methanol + other soluble) divided by dpm from a direct addition of soluble NSR to scintillation cocktail. For samples in which the soluble NSR represented at least 5.0% of the total dpm in a bottle, the average degree of recovery from the HPLC analysis was 96.2% (coefficient of variation = 3.01%).

An abbreviated version of the Schmidt degradation was used to determine which of the acetate carbons was labeled, using the reaction vials described by Fuchs et al. (11). In order to minimize the presence of organic compounds other than HAc, samples were prepared by processing them through the HPLC and collecting only the HAc fraction. The procedure was carried through the splitting of HAc into  $\text{CO}_2$  and  $\text{CH}_3\text{NH}_2$ .  $\text{CO}_2$  (from the carboxyl carbon) was absorbed in NaOH, transferred to liquid scintillation cocktail and counted.  $\text{CH}_3\text{NH}_2$  (from the methyl carbon) was likewise transferred to liquid scintillation cocktail and counted. From a  $^{14}\text{CH}_3\text{COO}^-$  standard, nearly 93% was recovered as  $^{14}\text{CH}_3\text{NH}_2$  with only a trace of  $^{14}\text{CO}_2$ ; from a  $\text{CH}_3^{14}\text{COO}^-$  standard, nearly 91% was recovered as  $^{14}\text{CO}_2$  with only a trace of  $^{14}\text{CH}_3\text{NH}_2$ .

$^{14}\text{C}$  activity was assayed with a Beckman model 9800 liquid scintillation counter. Corrections for counting efficiency were made according to a quench curve (sample H# versus efficiency).

**Hydrogen.** Hydrogen levels were monitored with an RGA2 reduction gas detector (Trace Analytical; Menlo Park, CA). Headspace samples were injected onto a 1% SP-1000 Carbowax B column, described above. By using an automated switching valve timed to change at 1.5 minutes, the first compounds eluting off the Carbowax B column —  $\text{H}_2$ ,  $\text{CH}_4$ , and  $\text{CO}_2$  — were routed to a 60/80 Carbosieve G column (3.2-mm x 3.05-m stainless-steel; Supelco, Inc.), while any DCM that was present eluted later from the Carbowax column and was routed to the flame-ionization detector. On the Carbosieve G column,  $\text{H}_2$  was resolved from the other compounds before being sent to the reduction gas detector. The GC was operated isothermally at  $100^\circ\text{C}$ , for a total of 6 minutes, with a helium carrier gas flow rate of 30 ml/min.  $\text{H}_2$  eluted at approximately 1.25 minutes. Standards were prepared in serum bottles containing 100 ml of

distilled deionized water, which were then purged with  $N_2$  (about 60 ml/min for 5 min) and capped with grey-butyl septa, prior to adding  $H_2$ .

**Biomass.** Biomass was determined by measuring suspended organic carbon (SOC), calculated as total organic carbon in unfiltered minus filtered (0.45  $\mu m$ ) samples. A Model 700 TOC Analyzer (OI Corporation; College Station, TX) was used to measure organic carbon (1-ml samples, duplicate injections) with sodium persulfate serving as the oxidant (100 mg/sample); its effectiveness was confirmed by the method of standard additions.

## RESULTS

**Stoichiometry of DCM degradation and methane formation.** Results for one of the first-generation bottles (A-1) are shown in Figure 1. The initial DCM addition of 8  $\mu mol$  was consumed after a lag period of about 10 days. Subsequent 8-11  $\mu mol$  DCM additions were also degraded, with little or no lag. Chloromethane appeared in only trace amounts over the first 46 days. By day 84, losses of DCM from duplicate water control and autoclaved-inoculated control bottles were only 5.74% and 10.8%, respectively. Thus, the much faster disappearance of DCM in the inoculated bottle was a consequence of biological activity, rather than abiotic processes. Similar results for water controls and autoclaved-inoculated controls were obtained in all subsequent experiments. Over the longest period of time a set of controls was monitored (707 days), only 17.2% of the DCM was lost from water controls, 25.7% from autoclaved-inoculated controls.

After a long lag period (64 days), a second-generation enrichment culture (A-2) began to degrade repetitive 10-11  $\mu mol$  additions of DCM within three or four days. Prior to each DCM addition, 4 ml of mixed culture was withdrawn and replaced with 4 ml of new basal medium containing 50 mg/liter HAc. This mode of operation continued until day 193, after which only DCM-saturated water was added. On day 197, monitoring of cumulative DCM degradation and  $CH_4$  formation began. (It was necessary to purge the bottle's headspace of accumulated methane

before doing so, in order to keep CH<sub>4</sub> output below the upper detection limit of the flame-ionization detector.)

The absence of HAc additions did not deter DCM degradation. Between days 197 and 215, six additions of DCM (10-11  $\mu$ mol/addition) were degraded, totaling 61.3  $\mu$ mol of DCM consumed. Over the same 17-day interval, 31.4  $\mu$ mol of methane was produced, or 0.51 mol of methane produced per mol of DCM consumed. DCM additions were then stopped, while monitoring of methane production continued. Between days 215 and 217, 1.82  $\mu$ mol of methane was produced; only 0.94  $\mu$ mol additional methane was produced over the next six days (Figure 2). Thus, the absence of DCM resulted in a levelling off of methane output. This suggested that the CH<sub>4</sub> produced in the presence of DCM was a consequence of DCM degradation, rather than from some other electron donor (e.g., yeast extract or HAc) possibly left over from previous operation of the bottle.

DCM was added again on day 223. An acclimation period was observed before a rapid rate of DCM consumption resumed. When it did, DCM consumption and methane production were once again linked. With 17 cycles of DCM consumption completed, the slope of the best fit line through the data indicated 0.492 mol of methane produced per mol of DCM consumed ( $R^2 = 99.1\%$ ). DCM additions were stopped on day 275, and methane output correspondingly levelled off a second time (Figure 2).

Analogous experiments correlating methane formation with DCM degradation were conducted in 11 other bottles to which DCM was added as the sole organic substrate. The duration of the studies varied, but in no instance was less than 8 days. One bottle received only DCM for more than 300 days (151 repetitive additions, totaling 1.575 mmol DCM), with no indication of a need for any non-DCM organic-carbon or electron-donor source. As shown in Table 1, similar results were obtained, with the overall average being 0.473 mol of methane produced per mol of DCM consumed.

**Effect of inhibiting methanogenesis on DCM degradation.** In several bottles used to arrive at the above ratio, subsequent addition of 0.5-1.5 mM BES did not slow the

cultures' ability to continue degrading DCM. This amount of BES caused a partial inhibition of methanogenesis — methane output was not completely stopped, but was considerably below the above ratio. For example, in bottle B-13, 0.499 mol of  $\text{CH}_4$  was produced per mol of DCM degraded prior to BES addition. Following two 50- $\mu\text{mol}$  BES additions, DCM degradation continued unabated (about 10  $\mu\text{mol}$  per bottle every other day), but  $\text{CH}_4$  output was reduced by 86% compared to pre-BES addition (Figure 3). In bottles B-11 and B-12, similar results were obtained, with 74% and 84% reduction in methanogenesis following BES addition, respectively.

In several other bottles described in Table 1, subsequent addition of 50-55.5 mM BES completely stopped  $\text{CH}_4$  production, but the cultures continued to degrade DCM. For example, in bottle B-16, 0.469 mol of  $\text{CH}_4$  was produced per mol of DCM degraded (Figure 3). Addition of 5 mmol BES completely stopped  $\text{CH}_4$  production. DCM degradation was temporarily slowed, with eight days required to degrade 9.1  $\mu\text{mol}$  of DCM instead of two days. Lower initial DCM doses were subsequently added and then gradually increased until the previous rate (about 10-12  $\mu\text{mol}$  DCM degraded every other day) was restored and maintained for 20 days. Results for B-14 and B-15 were similar.

Concurrent with the addition of 5 mmol BES was accumulation of ethylene. Ethylene formation was also noted in bottles subjected to lower doses of BES, but only in trace amounts. The probable source of ethylene was degradation of BES, as has been observed by Belay and Daniels (1) in pure cultures of methanogens exposed to BES.

**Effect of vancomycin on DCM degradation.** Four bottles (C-1 through C-4) were prepared identically and continuously shaken in an orbital water bath. Over the first 29 days of operation, the ability of each bottle to repetitively degrade increasing levels of DCM was established. On day 29, C-1 and C-2 received 10 mg of vancomycin, while C-3 and C-4 continued to receive only DCM. As shown in Figure 4, C-3 was able to repetitively degrade increasing levels of DCM, up to 120  $\mu\text{mol}$  per bottle on day 41; no effort was made to increase the DCM dose above this, although in other cultures amounts twice as high were readily degraded. DCM degradation in C-1 continued at a rapid rate for only one spike after adding the vancomycin,

then declined considerably. Despite the tailing off in DCM degradation,  $\text{CH}_4$  increased in C-1 by 67  $\mu\text{mol}$  between days 29 and 49. Virtually identical results were obtained from bottles C-2 and C-4.

**Hydrogen formation during DCM degradation.** The possible appearance of  $\text{H}_2$  as a product of DCM degradation was evaluated in bottles B-19, B-20, and B-22. While they were continuously shaken in an orbital water bath, the amount of DCM added (as sole organic substrate) was gradually increased from approximately 10 to 200  $\mu\text{mol}$  per bottle every other day, then maintained at that level for at least 18 days prior to monitoring  $\text{H}_2$ . At these high DCM levels, HAc accumulated in all three bottles, though HAc was not fed (data not shown).

Results for B-22 are shown in Figure 5. Prior to DCM addition, the background level of  $\text{H}_2$  was less than 20 nmol per bottle ( $8 \times 10^{-6}$  atm). Following DCM addition,  $\text{H}_2$  accumulated as DCM degraded, reached a peak of 9.5  $\mu\text{mol}$  per bottle, then returned to background levels.  $\text{CH}_4$  increased as  $\text{H}_2$  accumulated, then leveled off when the  $\text{H}_2$  returned to background levels. Results for B-19 and B-20 were similar; the peak  $\text{H}_2$  levels reached were 11.3 and 1.5  $\mu\text{mol}$  per bottle, respectively.

The average rates of DCM disappearance were 6.23, 6.19, and 7.65  $\mu\text{mol hour}^{-1}$  bottle $^{-1}$  in B-19, B-20, and B-22, respectively. Biomass levels were measured prior to DCM addition and after it was degraded; no significant change occurred, with an average of 4.51, 4.59 and 4.16 mg SOC per bottle in B-19, B-20, and B-22, respectively. The reason for the higher DCM utilization rate in B-22 is unknown; it had been in operation approximately one-third as long as the other two bottles.

Operation of B-19, B-20 and B-22 at high DCM doses resulted in partial inhibition of methanogenesis, just as low doses (0.5-1.5 mM) of BES did in other bottles. For example, when 200  $\mu\text{mol}$  of DCM was added to B-22, it was degraded below the detection limit in 27.5 hours (Figure 5). Twenty hours later, cumulative methane output was only 7  $\mu\text{mol}$ , or 0.035 mol  $\text{CH}_4/\text{mol}$  DCM; this is 93% lower than the ratio observed when cultures received only 10-12  $\mu\text{mol}$  DCM every other day (Table 1).



**Biodegradation pathway analysis using [ $^{14}\text{C}$ ]DCM.** The fate of [ $^{14}\text{C}$ ]DCM added to bottles B-1 through B-18 is presented in Table 2. These results have been corrected for the small amount of  $^{14}\text{CO}_2$  present in the [ $^{14}\text{C}$ ]DCM stock solution (averaging 2.7%, as indicated by analysis of the water controls), and represent the percent of total dpm *recovered* in each bottle. On average, 96.5% (coefficient of variation = 4.2%) of the dpm added (measured by direct addition of [ $^{14}\text{C}$ ]DCM stock to scintillation cocktail) was recovered [as DCM +  $\text{CH}_4$  +  $\text{CO}_2$  + HAc + methanol + other soluble NSR + nonsoluble NSR (assumed to be biomass)]. Recoveries from water controls also averaged 96.5%.

Four general cases are represented in Table 2, based on the condition of methanogenesis. In the first, methanogenesis was uninhibited (B-1 through B-8), with varying levels of HAc added along with DCM. In the second, methanogenesis was partially inhibited with high doses of DCM (B-9 and B-10). In the third, methanogenesis was partially inhibited with low doses of BES (B-11, B-12, and B-13). In the fourth, methanogenesis was completely inhibited using high doses of BES (B-14 through B-18). The following major results emerged:

Under all of the conditions examined the principal product of [ $^{14}\text{C}$ ]DCM degradation was  $^{14}\text{CO}_2$ , representing at least 61% of the  $^{14}\text{C}$  recovered.  $^{14}\text{CH}_4$  was formed in appreciable amounts (9%-36%) only when methanogenesis was uninhibited. The higher the amount of HAc added with each dose of DCM, the higher the percentage of  $^{14}\text{CH}_4$ . When methanogenesis was partially inhibited, with either high doses of DCM or low doses of BES, little or none of the methane that was produced was radiolabeled. As would be expected, no  $^{14}\text{CH}_4$  was measured when methanogenesis was completely inhibited with BES.

When methanogenesis was partially inhibited, the most important product after  $^{14}\text{CO}_2$  was [ $^{14}\text{C}$ ]HAc (20-30%). Partial inhibition of methanogenesis in B-9 and B-10 (no BES added) was caused by high doses of DCM, just as it was in B-19, B-20 and B-22 (described above). Between days 140 and 150, when [ $^{14}\text{C}$ ]DCM was added every other day, B-9 degraded 0.986 mmol of DCM and produced 0.140 mmol of  $\text{CH}_4$ , or 70% less than the ratio reported in Table 1. Over the

same interval B-10 degraded 1.114 mmol of DCM and produced 0.067 mmol of  $\text{CH}_4$ , amounting to an 87% inhibition of methanogenesis.

When methanogenesis was completely inhibited, the most important product after  $^{14}\text{CO}_2$  was also  $[^{14}\text{C}]\text{HAc}$  (22-41%). Bottles B-14, B-15, and B-16 received low doses of DCM and a single addition of  $[^{14}\text{C}]\text{DCM}$ , while B-17 and B-18 received higher doses of DCM and five additions of  $[^{14}\text{C}]\text{DCM}$ .

When BES was added to cultures, the next most important product after  $^{14}\text{CO}_2$  and  $[^{14}\text{C}]\text{HAc}$  was  $^{14}\text{CH}_3\text{OH}$ . On average, the higher the amount of BES, the higher the amount of  $^{14}\text{CH}_3\text{OH}$ . The "other soluble" category — never of much consequence under any of the conditions examined — tended to be slightly higher when methanogenesis was partially or totally inhibited. A relatively small amount of the  $^{14}\text{C}$  label (0.23-2.84%) was recovered as nonsoluble NSR, which was assumed to be biomass.

Schmidt degradation of HAc produced in two bottles was used to determine the distribution of the label on the carbons. In B-11 ( $[^{14}\text{C}]\text{DCM}$  added when methanogenesis was partially inhibited with BES), HAc represented 19.6% of the total dpm recovered; in B-14 ( $[^{14}\text{C}]\text{DCM}$  added when methanogenesis was completely inhibited with BES), HAc represented 22.5% of the total dpm recovered. In both bottles, replicate analyses indicated that the label was almost entirely (>94%) on the methyl carbon of HAc.

**Use of DCM as a growth substrate.** Figure 6a presents results for bottles receiving DCM (B-9 and B-10) and the associated controls receiving no DCM. There was no statistically significant change in SOC levels over time in the controls; their overall average was 40.9  $\mu\text{mol}$  SOC per bottle. However, SOC in B-9 and B-10 rose definitively above the controls beginning on day 49, just as cumulative DCM consumption began to rise significantly (Figure 6b). The accumulation of SOC and increase in cumulative DCM degradation continued through day 77, at which point the rate of DCM degradation and SOC formation slowed markedly. The likely reason was a significant drop in pH, presumably as a consequence of the HCl produced from DCM biotransformation; prior to day 77, B-9 and B-10 were dechlorinating more than 200  $\mu\text{mol}$  DCM

per bottle every other day. On day 91, the pHs were 5.10 in B-9 and 5.44 in B-10, versus 7.37 and 7.56 in the controls.  $\text{NaHCO}_3$  was added to B-9 and B-10 in order to raise the pH to 7.0-7.7 and they — as well as the controls — were reinoculated with 5 ml of a culture actively degrading DCM. This restoration effort was successful. On day 91 the rate of DCM degradation was about 35  $\mu\text{mol}$  per bottle every other day; this was gradually built up to more than 160  $\mu\text{mol}$  by day 111. Starting on day 105, appropriate amounts of 8 M NaOH were added along with DCM in order to maintain the pH in B-9 and B-10 between 6.5 and 7.0.

SOC formation did not respond immediately to the restoration of rapid DCM degradation. Between days 77 and 119, the SOC in B-9 actually decreased slightly, then rose well above the day 77 level on day 133. In B-10, a similar decrease occurred through day 105, but consecutive increases in SOC over the day 77 level were observed on days 119 and 133 (Figure 6a).

Before the decrease in pH, SOC formation and DCM degradation in B-9 and B-10 were highly correlated, with the slope of the best fit line indicating 0.085 g biomass carbon formed per g DCM carbon consumed (Figure 7). This translates to an observed yield of 0.060 grams of biomass (dry weight organic matter) formed per gram of oxygen demand consumed, assuming a typical composition for bacterial cell organic matter of  $\text{C}_5\text{H}_7\text{O}_2\text{N}$  (18) and 32 g oxygen demand per mol of DCM.

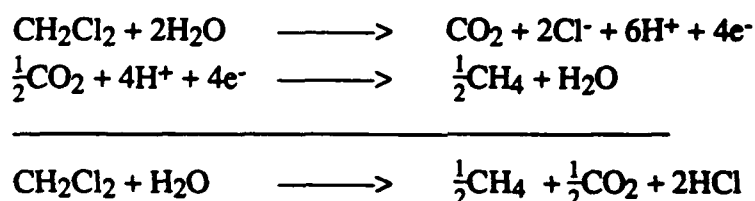
Figure 8 shows that the observed methane output from B-9 and B-10 was well below the amount that could have been produced, assuming 0.5 mol of methane per mol of DCM degraded (plus  $\text{CH}_4$  from the controls). By day 133, methane output in B-9 and B-10 was only 16.8% and 12.6%, respectively, of the potential methane output. This partial inhibition of methanogenesis was a consequence of the high levels of DCM added during the growth experiment.

The fraction of observed growth attributable to methanogens and nonmethanogens was estimated based on the amount of methane produced, since methanogens presumably cannot grow without producing methane (19). By day 77 net methane formation in B-9 was 237  $\mu\text{mol}$ , equivalent to 15.2 mg of oxygen demand used for energy (1 mmol  $\text{CH}_4$  = 64 mg oxygen demand), or 1.90 milli-electron equivalents [8 mg oxygen demand = 1 milli-electron equivalent (23)]. For

CO<sub>2</sub>-reducing methanogens, the ratio of electron equivalents used for energy to electron equivalent of cells formed is approximately 12.5 (the ratio is even higher for acetoclastic methanogens) (23). Therefore, 0.152 milli-electron equivalent of biomass was formed as a consequence of methanogenesis (1.90/12.5); this amounts to 0.859 mg biomass, assuming C<sub>5</sub>H<sub>7</sub>O<sub>2</sub>N describes the composition of bacterial cell organic matter [1 electron equivalent = 0.05 C<sub>5</sub>H<sub>7</sub>O<sub>2</sub>N = 5.65 g biomass (18, 23)]. By day 77 net biomass formation in B-9 was 222 µmol SOC, or 5.02 mg of biomass. Thus, in B-9 only 17.1% (0.859/5.02) of the net biomass formed can be credited to methanogens. The same calculations applied to B-10 indicate only 15.1% of the net biomass formed can be credited to methanogens, the balance to nonmethanogens.

## DISCUSSION

The experimentally determined ratio of methane formed to DCM consumed (Table 1) was very close to the ratio expected (0.5 mol CH<sub>4</sub>/mol DCM) based on stoichiometric conservation of electrons:



This relationship was followed only when the DCM dose was low enough ( $\leq 12$  µmol per bottle) to avoid inhibition of methanogenesis. A ratio slightly less than 0.5 is consistent with the finding that DCM serves as a growth substrate.

The accumulated evidence suggests that nonmethanogens were responsible for DCM degradation. First, cultures continued to degrade DCM after methanogenesis was completely inhibited by BES. Second, vancomycin, an antibiotic that prevents cell-wall synthesis in eubacteria by interfering with the formation of the UDP-MurNAc-pentapeptide precursors of murein (3), essentially stopped DCM degradation. Third, when DCM was provided to cultures as the sole organic compound, the majority of the growth observed was attributable to

nonmethanogens. And fourth, the formation of HAc (a major product of DCM degradation) from  $C_1$  compounds is characteristic of acetogenic bacteria (21). Although Westermann et al. (36) recently demonstrated that methanogens are also capable of synthesizing acetate from  $C_1$  substrates ( $H_2$ - $CO_2$  and methanol), the other evidence tends to rule out their possible role in DCM degradation.

A proposed model for DCM degradation under methanogenic conditions is presented in Figure 9. The principal mode of DCM degradation under all conditions examined was oxidation to  $CO_2$ ; the organisms mediating this reaction have therefore been termed "DCM oxidizers." The other major mode of DCM transformation was fermentation to acetic acid, with the methyl carbon coming directly from DCM and the carboxyl carbon coming from unlabeled  $CO_2$ , available in the large pool of carbonates present in the basal medium. DCM degradation is thus a disproportionation: a portion of the DCM is oxidized, making reducing equivalents available for reduction of an equal amount of DCM.

Methanogens consumed the products of DCM degradation.  $CO_2$ -reducing methanogens used some of the electrons made available from DCM oxidation to form methane. At least a portion of those electrons were available in the form of  $H_2$  (Figure 5). Recent evidence indicates that the majority of electron transfer in methanogenic systems occurs via formate rather than  $H_2$  (4, 33), but this possibility was not explored with the DCM-degrading cultures. Methane formed by  $CO_2$ -reducers was unlabeled because the  $^{14}CO_2$  formed from oxidation of  $^{14}CH_2Cl_2$  was essentially diluted out by the large unlabeled carbonate pool in the basal medium. Aceticlastic methanogens produced methane from the acetic acid formed by fermentation of DCM. This route was the probable source of  $^{14}CH_4$ ; methane from aceticlastic methanogens was derived from the methyl carbon of HAc, which was shown to be the carbon carrying the  $^{14}C$ -label from DCM.

Figure 9 also shows where inhibitors exerted their effects. DCM degradation was stopped by vancomycin. We did not test the specificity of vancomycin on the DCM-degrading enrichment cultures. However, Murray and Zinder (24) reported that 100 mg/liter of vancomycin (the dose used in this study) inhibits eubacteria without effecting Archaeobacteria, specifically methane

production or growth of *Methanosarcina barkeri* strain 227. Our data indicate qualitatively that vancomycin did not totally stop methanogenesis. As shown in Figure 4, methane production continued after the addition of vancomycin. A possible source of the methane formed was previously accumulated acetate.

Inhibition of methanogenesis is also indicated in Figure 9. With low doses of BES (0.5-1.5 mM) and high doses of DCM (about 2 mM), methane production was significantly reduced (i.e., partially inhibited), but not stopped (Figure 3). However, of the methane that was formed, none was labeled (Table 2). This result is consistent with the hypothesis that  $^{14}\text{CH}_4$  came from  $^{14}\text{CH}_3\text{COOH}$  and not  $^{14}\text{CO}_2$ , since it appears that the acetoclastic methanogens were inhibited to a much greater extent than the  $\text{CO}_2$ -reducing methanogens by low levels of BES and high doses of DCM. Less than stoichiometric formation of methane and accumulation of acetate (but not  $\text{H}_2$ ) were observed in all cultures receiving repetitive high doses of DCM, such as those used in the growth experiments (9). Zinder et al. (38) also reported that a low dose of BES (1.0 mM) inhibited acetoclastic methanogenesis whereas a much higher dose (50 mM) was required to completely inhibit  $\text{CO}_2$ -reduction; the same level was used in this study to completely inhibit methanogenesis.

The recently elucidated "acetyl-CoA pathway" (21) makes it possible to speculate on how DCM might enter the metabolic pathway of acetogenic bacteria. Since DCM is incorporated into the methyl carbon of acetate, DCM probably enters at the level of one of the tetrahydrofolates, such as hydroxymethyltetrahydrofolate. Transformation of DCM to hydroxymethyltetrahydrofolate may occur by a mechanism similar to that described for *Hyphomicrobium* DM2 (5), i.e., dechlorination to a bound chloromethyl compound (mediated by a dehalogenase), followed by nonenzymatic hydrolytic formation of a bound hydroxymethyl compound. The reducing power needed ( $4e^-$ ) to complete formation of acetate from hydroxymethyltetrahydrofolate would come from oxidation of another mole of DCM to  $\text{CO}_2$ . An analogous situation has been described for synthesis of acetate from methanol, the methyl group of which is incorporated directly into the methyl group of acetate.

Methanol appears to enter the acetyl-CoA pathway at the level of the protein-bound Co-methylcorrinoid (21).

It is less clear how DCM oxidation might occur, be it in the same or a different organism. If an acetogen does carry out oxidation, the pathway may be similar to that for methanol. Some of the methanol consumed by acetogens must be oxidized to  $\text{CO}_2$  to provide the reducing equivalents needed for formation of the methyl carbon of acetate (21); the same holds for DCM.

As shown in Table 3, the thermodynamics of DCM degradation are very favorable, in part because of the substantial amount of free energy associated with removal of  $\text{Cl}^-$ . Half reactions were calculated from  $\Delta G^\circ_f$  data [all values except DCM from Stumm and Morgan (31); DCM (g) from Reid et al. (26), converted to DCM (aq) using a Henry's constant of 2.135 liter-atm/mol at 25°C (14)].  $\Delta G$  values were calculated from  $\Delta G^\circ$ , assuming the following "typical" conditions:  $\text{CO}_2 = 0.3$  atm,  $\text{H}_2 = 10^{-4}$  atm,  $\text{Cl}^- = 37$  mM,  $\text{H}^+ = 10^{-7}$  M,  $\text{HCO}_3^- = 59.5$  mM,  $\text{CH}_3\text{COO}^- = 0.5$  mM,  $\text{CH}_3\text{OH} = 31.3$   $\mu\text{M}$ , and DCM = 2.0 mM. The oxidation of DCM to  $\text{CO}_2$  plus  $\text{H}_2$  (reaction 1) remains favorable even if a hydrogen partial pressure of 1 atm is assumed.

One of the products observed from [ $^{14}\text{C}$ ]DCM degradation not included in Figure 9 is  $^{14}\text{CH}_3\text{OH}$ , which tended to accumulate significantly (i.e., >1% of total dpm) only when BES was used to inhibit methanogenesis (Table 2). The source of the methanol formed is not known. Some of the possibilities, shown in Table 3, include directly from DCM (reaction 4), synthesis from  $\text{H}_2/\text{CO}_2$  (reaction 5), or from acetate (reaction 6). Under the typical conditions experienced by the enrichment cultures, the only thermodynamically favorable possibility is methanol formation from DCM (reaction 4). Since BES promoted  $^{14}\text{CH}_3\text{OH}$  formation, it appears that BES had some effect on the DCM-degrading organism. The rate of DCM degradation in enrichment cultures exposed to 50-55.5 mM BES was approximately one-half that of cultures without BES. No reports were found in the literature regarding the effect of BES on acetogens. However, formation of methanol by an acetogen from a single carbon substrate has been demonstrated previously, using *Clostridium thermoaceticum* in the presence of carbon monoxide and methylviologen (37).

Biotransformation under methanogenic conditions of several other chlorinated aliphatics in widespread use (e.g., tetrachloroethylene and trichloroethylene) occurs primarily by reductive dechlorination. This process requires the supply of an external electron donor (8, 10). With DCM, the principal transformation pathways were oxidation to  $\text{CO}_2$  and fermentation to acetate, not reductive dechlorination; chloromethane never accumulated beyond trace levels, even in first-generation cultures previously unexposed to DCM (Figure 1). Furthermore, DCM degradation was sustained for extended periods without the need for an external electron donor.

The results of this research appear to be the first demonstration of growth under methanogenic conditions using a chlorinated aliphatic compound as the sole organic carbon and energy source. Other studies have shown that growth on a chlorinated organic supplied as the sole carbon and energy source is possible under aerobic conditions. For example, use of DCM as a growth substrate has been demonstrated in enrichment cultures (27) and by pure cultures of *Pseudomonas* and *Hyphomicrobium* species (5, 12, 20, 29). Other chlorinated alkanes shown capable of supporting growth of aerobic organisms include methyl chloride (17), vinyl chloride (16), and 1,2-dichloroethane (30).

For some very good reasons, most studies examining dehalogenation under anaerobic conditions have not addressed the question of whether or not the halogenated compound can serve as a growth substrate. Low concentrations (e.g.,  $\mu\text{g/liter}$  range) have usually been employed — often to avoid toxicity. However, very low concentrations are unlikely to support growth (and furthermore present difficulty in detecting growth, should it occur). An additional problem is presented in those instances where reductive dehalogenation is the predominant halogen removal mechanism, because an auxiliary electron donor is required to sustain the transformation. If the auxiliary electron donor supports growth apart from reductive dechlorination reactions (e.g., via methanogenesis, sulfate reduction, or fermentation), it becomes very difficult to ascertain whether energy is derived from dechlorination of the halogenated compound. With DCM, the lack a requirement for an auxiliary electron donor allowed relatively easy determination that this compound can serve as a growth substrate.



The principal products of DCM biotransformation under methanogenic conditions — CO<sub>2</sub> and HAc — are environmentally acceptable. Since the fate of other chlorinated solvents (including tetrachloroethylene, trichloroethylene, carbon tetrachloride, and chloroform) under methanogenic conditions often results in some residual hazardous transformation product(s), DCM may be viewed from an environmental standpoint as a "lesser evil." To the extent that chlorinated solvents remain in widespread use, the specific ones employed should be restricted to those that have the least hazardous environmental fate.

### ACKNOWLEDGEMENTS

This research was supported by the U.S. Air Force Engineering and Services Center (AFESC), Tyndall AFB, FL, under contract no. F08635-86-C-0161.

The authors gratefully acknowledge the assistance of Stephen H. Zinder, Department of Microbiology, Cornell University, who first suggested the possibility of acetogenic activity in our DCM-degrading enrichment cultures. Anthony Alexander, Cornell University Mass Spectrometry Facility, and Mike Henley, U.S. Air Force Engineering and Services Center, provided GC-MS support.

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TABLE 1: Correlating Methane Production and DCM Degradation<sup>a</sup>

Bottle no.	n	Total CH <sub>4</sub> Produced (μmoles)	Total DCM Consumed (μmoles)	Slope	
				CH <sub>4</sub> /DCM	R <sup>2</sup> (%)
A-2	17	85.5	173	0.492	99.1
B-7	12	62.7	131	0.483	99.9
B-8	11	66.4	121	0.551	99.5
B-11	4	22.8	43.5	0.521	99.8
B-12	22	104	241	0.428	99.8
B-13	15	80.1	165	0.499	99.8
B-14	56	284	617	0.453	99.9
B-15	10	55.6	118	0.456	99.7
B-16	12	69.3	140	0.469	99.0
B-19	108	547	1150	0.477	100.0
B-20	151	763	1580	0.477	100.0
B-21	46	240	518	<u>0.458</u>	99.9

*weighted average* = 0.473

<sup>a</sup>All bottles received DCM as sole organic substrate, typically 10-12 μmoles/bottle every other day; n = number of points included in the regression analysis of CH<sub>4</sub> produced versus DCM consumed; each repetitive addition of DCM represented one point; the average slope was weighted according to n.

TABLE 2: Distribution of  $^{14}\text{C}$  from Degradation of  $[^{14}\text{C}]\text{DCM}$ 

----- percent of total dpm recovered as: -----							
Bottle	DCM	CH <sub>4</sub>	CO <sub>2</sub>	<u>Soluble NSR</u>			Biomass
				HAc	MeOH	Other	
<i>Methanogenesis uninhibited, 10 mg HAc added with each dose of DCM:</i>							
B-1	0.85	32.5	64.1	0.14	0.11	0.31	2.02
B-2	0.98	36.1	60.5	0.16	0.12	0.24	1.91
average	0.92	34.3	62.3	0.15	0.12	0.28	1.97
<i>Methanogenesis uninhibited, 1 mg HAc added with each dose of DCM:</i>							
B-3	0.00	26.7	69.1	0.40	1.04	0.44	2.31
B-4	0.81	28.7	66.9	0.63	0.00	0.49	2.45
average	0.41	27.7	68.0	0.52	0.52	0.47	2.38
<i>Methanogenesis uninhibited, 0.1 mg HAc added with each dose of DCM:</i>							
B-5	2.85	24.4	67.7	0.69	1.07	0.60	2.65
B-6	0.48	22.6	70.2	1.46	1.77	0.45	3.02
average	1.67	23.5	69.0	1.08	1.42	0.53	2.84
<i>Methanogenesis uninhibited, no HAc added:</i>							
B-7	2.97	18.2	73.6	0.74	0.00	0.63	3.83
B-8	1.03	9.09	81.5	5.83	0.00	0.63	1.89
average	2.00	13.7	77.6	3.29	0.00	0.63	2.86

continued . . .

TABLE 2, continued

----- percent of total dpm recovered as: -----							
Bottle	DCM	CH <sub>4</sub>	CO <sub>2</sub>	Soluble NSR			Biomass
				HAc	MeOH	Other	
<i>Methanogenesis partially inhibited with high DCM; no HAc added:</i>							
B-9	0.62	1.07	68.7	27.8	0.17	1.54	0.13
B-10	0.27	0.79	67.1	29.8	0.14	1.59	0.32
average	0.45	0.93	67.9	28.8	0.16	1.57	0.23
<i>Methanogenesis partially inhibited with BES; no HAc added:</i>							
B-11	0.54	0.076	73.4	19.6	4.78	0.27	1.30
B-12	0.07	0.00	69.0	26.4	2.13	1.44	1.03
B-13	0.59	0.029	72.1	23.2	2.41	1.28	0.40
average	0.40	0.035	71.5	23.1	3.11	1.00	0.91
<i>Methanogenesis completely inhibited with BES; no HAc added:</i>							
B-14	1.23	0.00	65.5	22.5	9.46	0.80	0.52
B-15	0.20	0.00	70.8	22.3	3.57	1.58	1.55
B-16	0.60	0.00	67.7	22.5	6.30	1.85	1.01
B-17	0.052	0.015	54.0	40.7	3.17	2.04	0.00
B-18	0.15	0.008	61.1	36.1	0.42	2.27	0.00
average	0.45	0.005	63.8	28.8	4.58	1.71	0.62



TABLE 3: Thermodynamic Considerations Relating to DCM Degradation (kcal/electron equiv.)

	$\Delta G^a$	$\Delta G^b$
<i>Half Reactions</i>		
1. $\frac{1}{4} \text{CH}_2\text{Cl}_2 + \frac{1}{2} \text{H}_2\text{O} = \frac{1}{4} \text{CO}_2 + \frac{1}{2} \text{Cl}^- + \frac{3}{2} \text{H}^+ + \text{e}^-$	-6.908	
2. $\frac{1}{2} \text{H}_2 = \text{H}^+ + \text{e}^-$	0.0	
3. $\frac{1}{8} \text{CH}_3\text{COO}^- + \frac{3}{8} \text{H}_2\text{O} = \frac{1}{8} \text{CO}_2 + \frac{1}{8} \text{HCO}_3^- + \text{H}^+ + \text{e}^-$	+2.981	
4. $\frac{1}{6} \text{CH}_3\text{OH} + \frac{1}{6} \text{H}_2\text{O} = \frac{1}{6} \text{CO}_2 + \text{H}^+ + \text{e}^-$	+0.725	
<i>Reactions</i>		
1. $\frac{1}{4} {}^*\text{CH}_2\text{Cl}_2 + \frac{1}{2} \text{H}_2\text{O} = \frac{1}{4} {}^*\text{CO}_2 + \frac{1}{2} \text{H}_2 + \frac{1}{2} \text{H}^+ + \frac{1}{2} \text{Cl}^-$	-6.91	-14.6
2. $\frac{1}{8} {}^*\text{CH}_2\text{Cl}_2 + \frac{1}{4} \text{H}_2 + \frac{1}{8} \text{HCO}_3^- =$ $\frac{1}{8} {}^*\text{CH}_3\text{COO}^- + \frac{1}{8} \text{H}_2\text{O} + \frac{1}{4} \text{H}^+ + \frac{1}{4} \text{Cl}^-$	-6.44	-7.84
3. $\frac{1}{4} {}^*\text{CH}_2\text{Cl}_2 + \frac{1}{8} \text{H}_2\text{O} + \frac{1}{8} \text{HCO}_3^- =$ $\frac{1}{8} {}^*\text{CH}_3\text{COO}^- + \frac{1}{8} {}^*\text{CO}_2 + \frac{1}{2} \text{H}^+ + \frac{1}{2} \text{Cl}^-$	-9.89	-15.2
4. $\frac{1}{6} {}^*\text{CH}_2\text{Cl}_2 + \frac{1}{6} \text{H}_2\text{O} + \frac{1}{6} \text{H}_2 = \frac{1}{6} {}^*\text{CH}_3\text{OH} + \frac{1}{3} \text{H}^+ + \frac{1}{3} \text{Cl}^-$	-5.33	-8.67
5. $\frac{1}{6} \text{CO}_2 + \frac{1}{2} \text{H}_2 = \frac{1}{6} \text{CH}_3\text{OH} + \frac{1}{6} \text{H}_2\text{O}$	-0.725	+1.10
6. $\frac{1}{8} \text{CH}_3\text{COO}^- + \frac{1}{4} \text{H}_2\text{O} = \frac{1}{8} \text{CH}_3\text{OH} + \frac{1}{8} \text{HCO}_3^- + \frac{1}{8} \text{H}_2$	+2.44	+1.34
7. $\frac{1}{8} \text{CO}_2 + \frac{1}{8} \text{HCO}_3^- + \frac{1}{2} \text{H}_2 = \frac{1}{8} \text{CH}_3\text{COO}^- + \frac{3}{8} \text{H}_2\text{O}$	-2.98	-0.519

$a$  25°C, 1 atm, with all species in their standard states at unit activity.

$b \Delta G = \Delta G^\circ + RT \ln \left[ \frac{[\text{C}]^c [\text{D}]^d}{[\text{A}]^a [\text{B}]^b} \right]$ , for the reaction,  $a\text{A} + b\text{B} \longrightarrow c\text{C} + d\text{D}$ ;

$R = 1.987 \times 10^{-3} \text{ kcal/mol} \cdot ^\circ\text{K}$ ,  $T = 298^\circ\text{K}$ .

## FIGURE LEGENDS

**Figure 1.** DCM (●) degradation and chloromethane (\*) formation in bottle A-1; DCM levels in duplicate water controls (Δ) and autoclaved-inoculated controls (□).

**Figure 2.** Cumulative DCM consumed and methane produced in bottle A-2 when DCM was the sole organic compound added.

**Figure 3.** Cumulative CH<sub>4</sub> formed and DCM consumed in bottles B-13 (Δ) and B-16 (●).

**Figure 4.** DCM degradation in bottle C-1 before and after adding vancomycin and in C-3, which received no vancomycin.

**Figure 5.** H<sub>2</sub> and CH<sub>4</sub> levels following addition of a high amount of DCM to bottle B-22.

**Figure 6.** Growth experiment results: DCM added, bottles B-9 (O) and B-10 (Δ); no DCM added, control bottles BC-9 (●) and BC-10 (▲).

**Figure 7.** Correlation between SOC formed and DCM consumed in bottles B-9 (O) and B-10 (Δ), for days 26-77 of the growth experiment. SOC formation was calculated as the measured SOC minus the overall average SOC in the controls.

**Figure 8.** Average CH<sub>4</sub> output during the growth experiment; DCM was added to bottles B-9 and B-10 (O); no DCM was added to control bottles BC-9 and BC-10 (Δ). Maximum potential methane output in B-9 and B-10 = {0.5 × (DCM consumed) + (CH<sub>4</sub> from controls)}.

**Figure 9.** Proposed model for DCM degradation under methanogenic conditions, including locations where inhibitors (high dose DCM, low dose BES, and high dose BES) exert their effects.

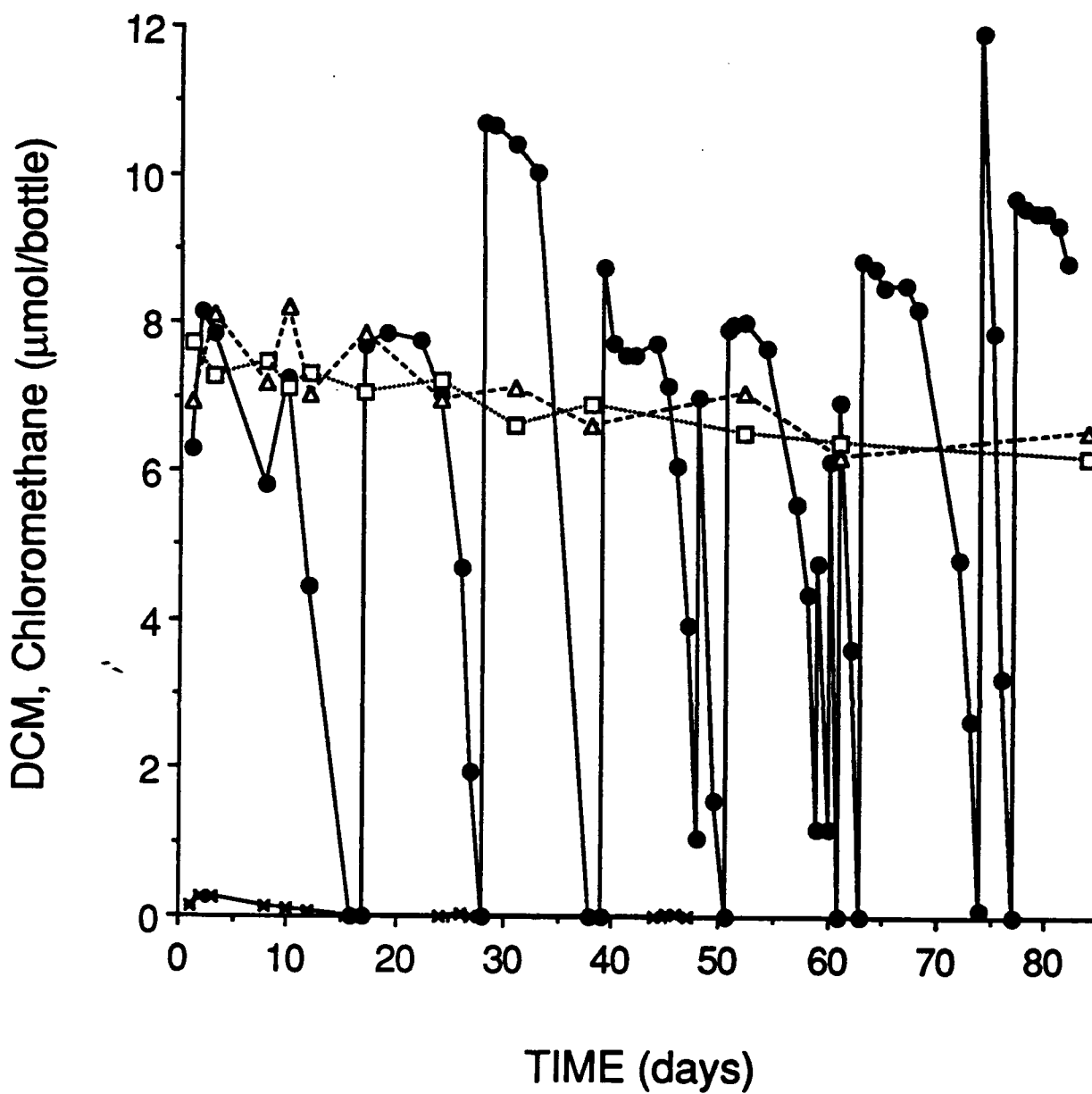


FIGURE 1

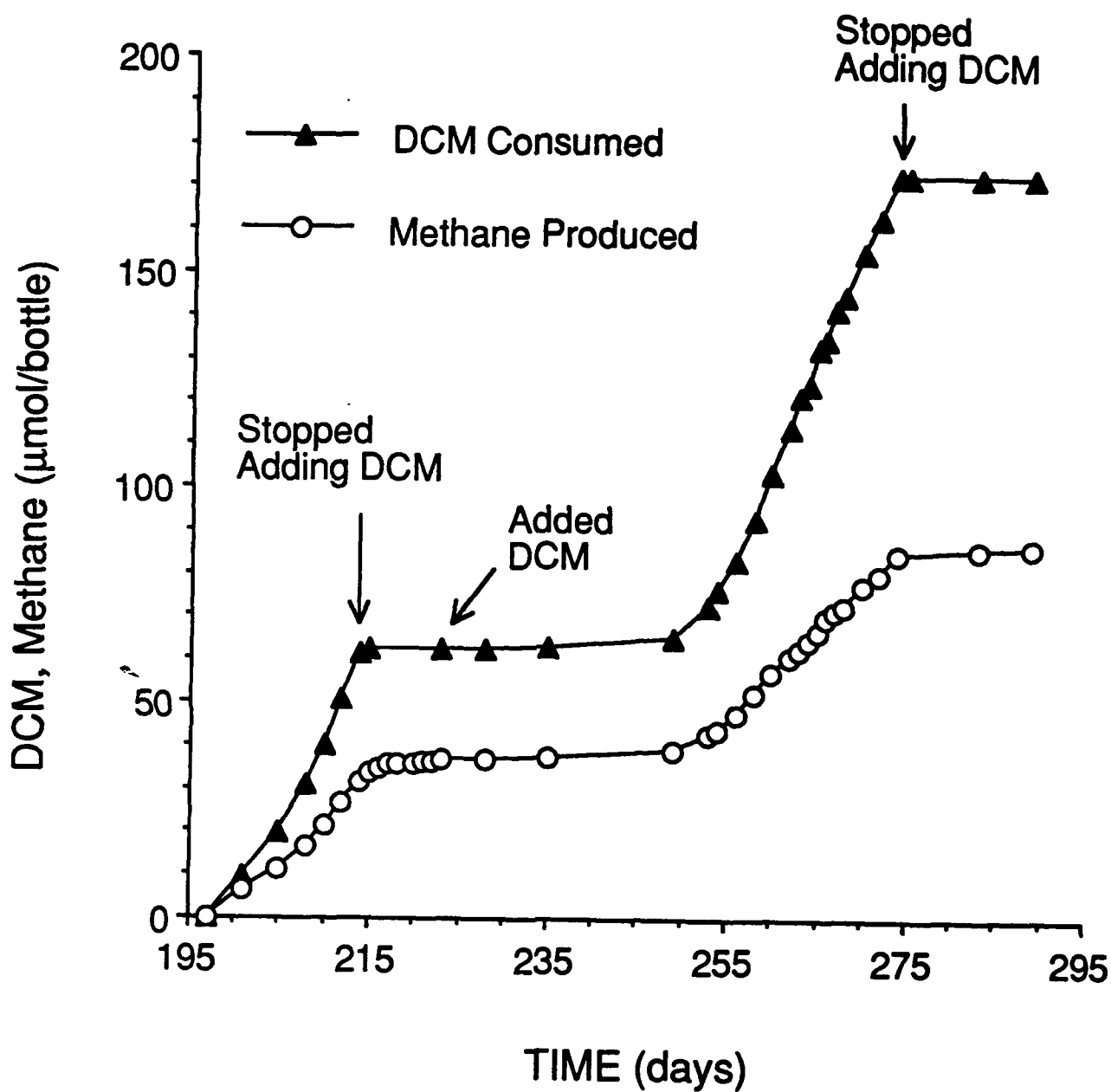


FIGURE 2

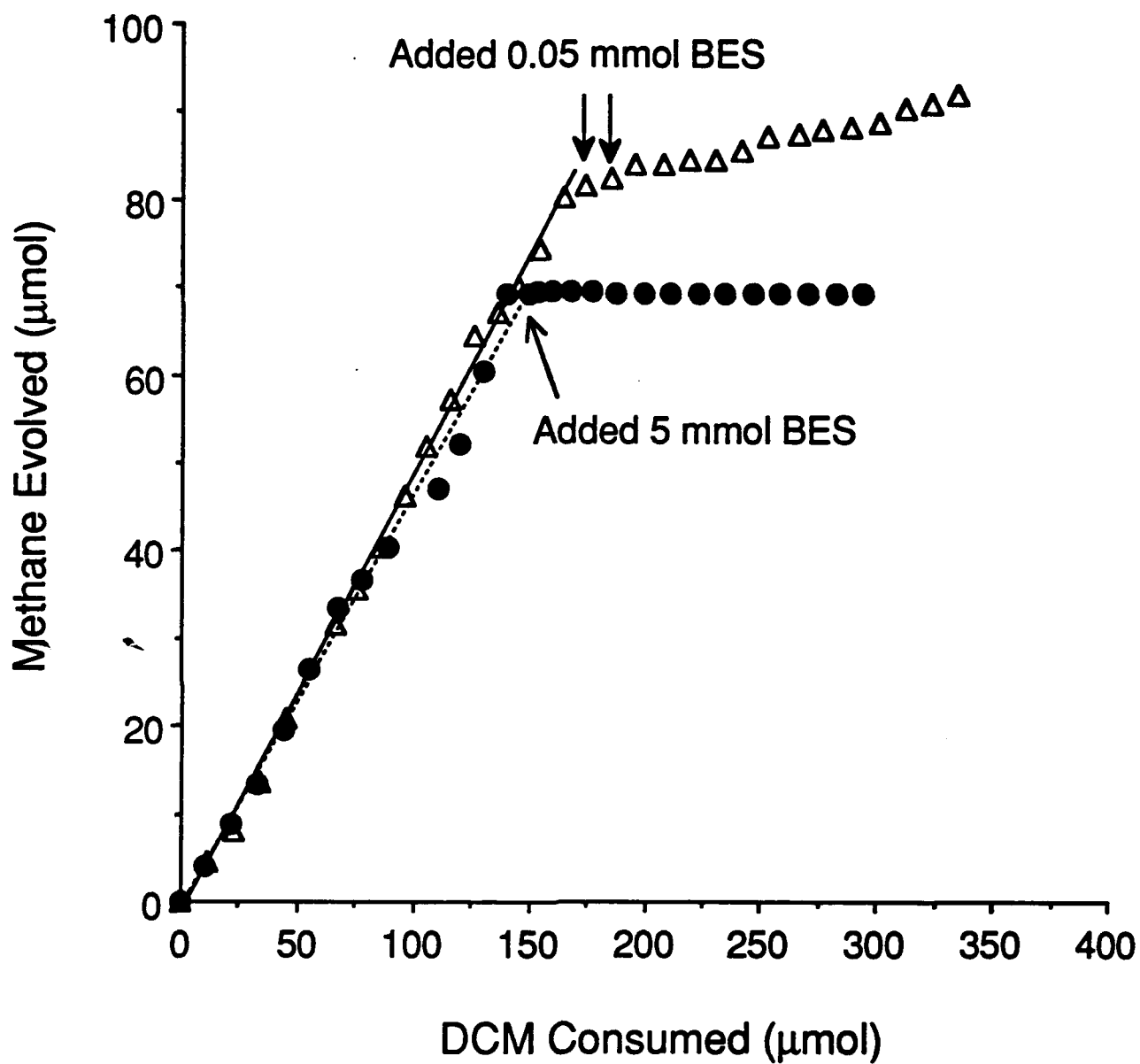


FIGURE 3

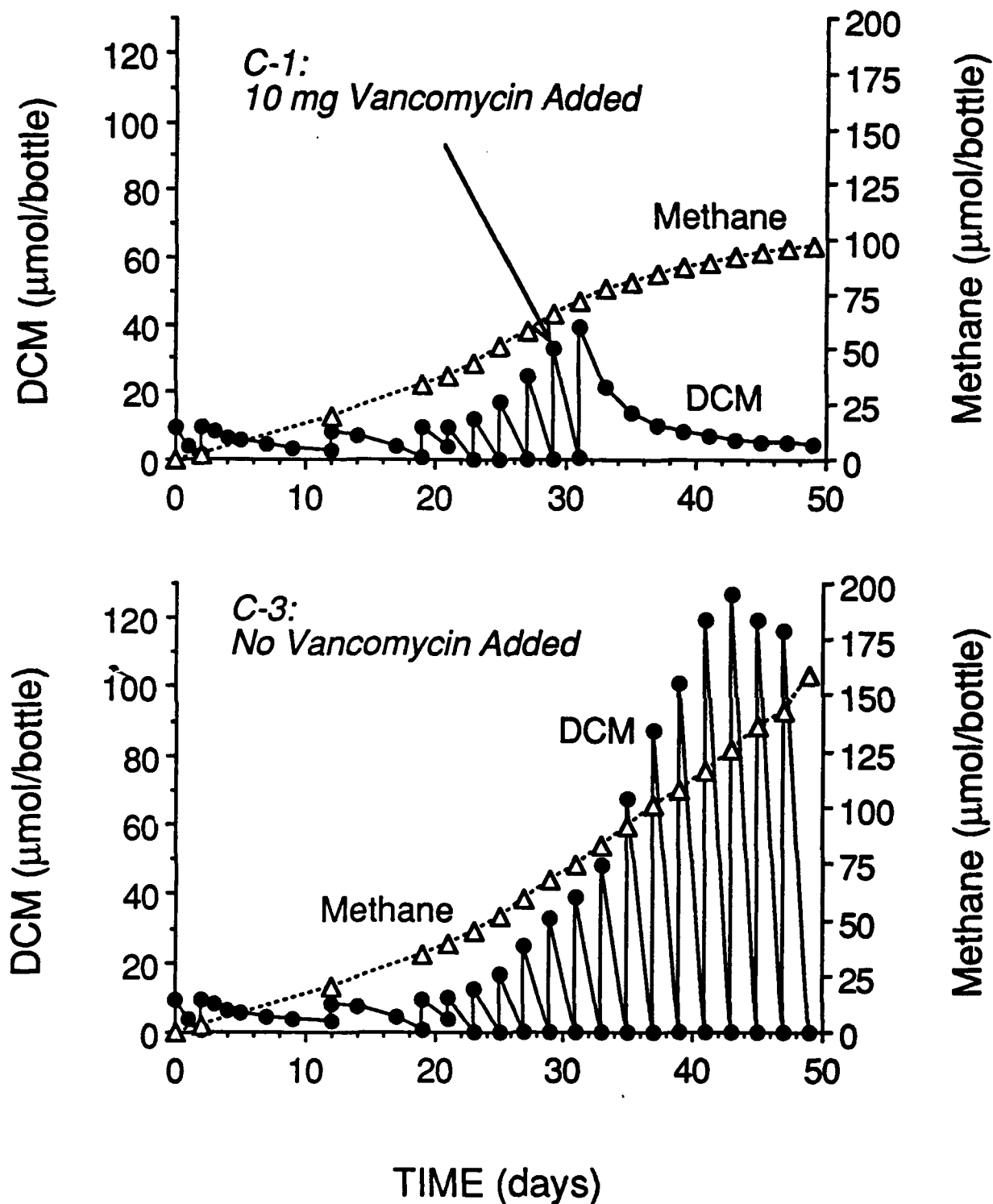


FIGURE 4

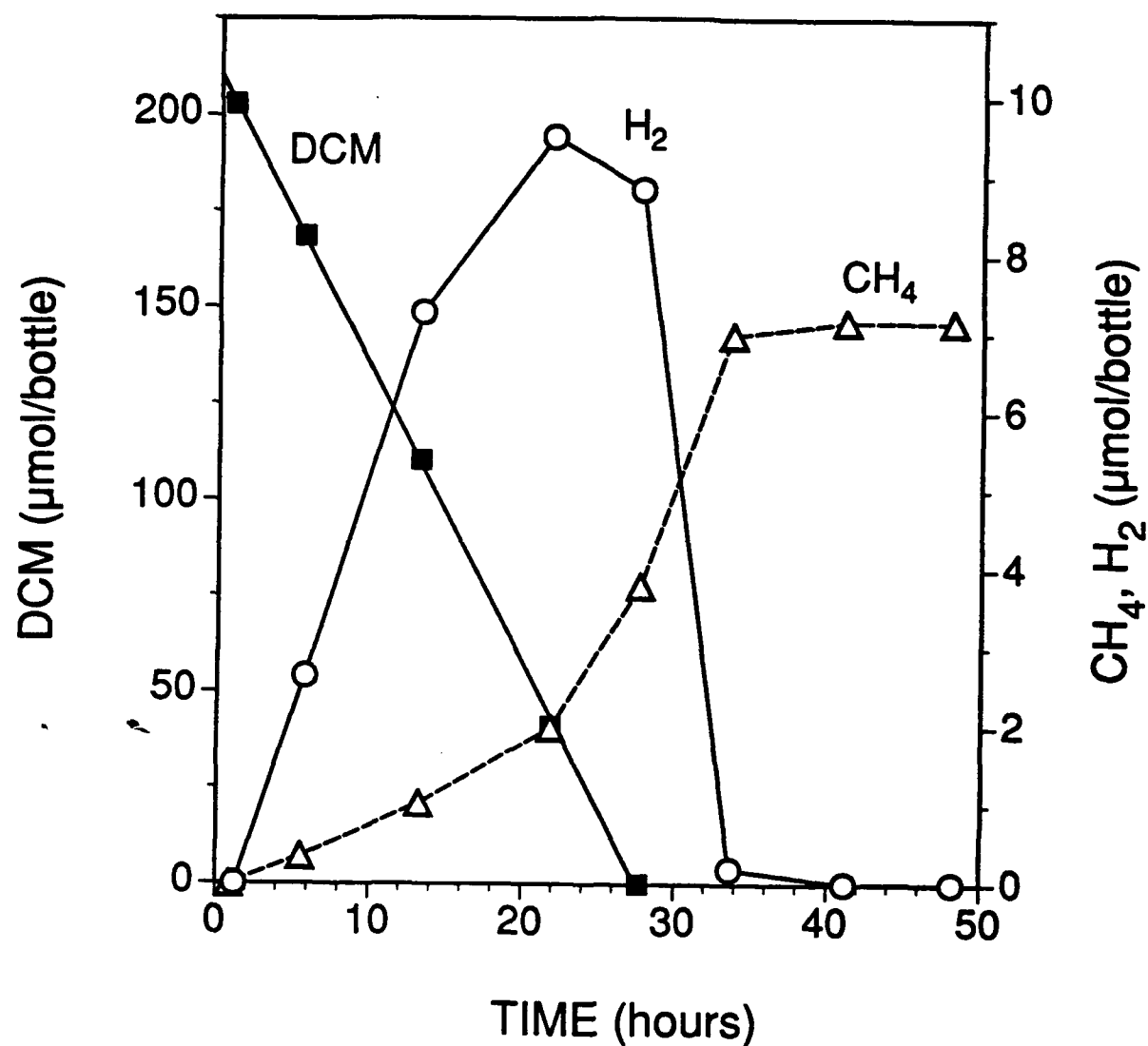


FIGURE 5

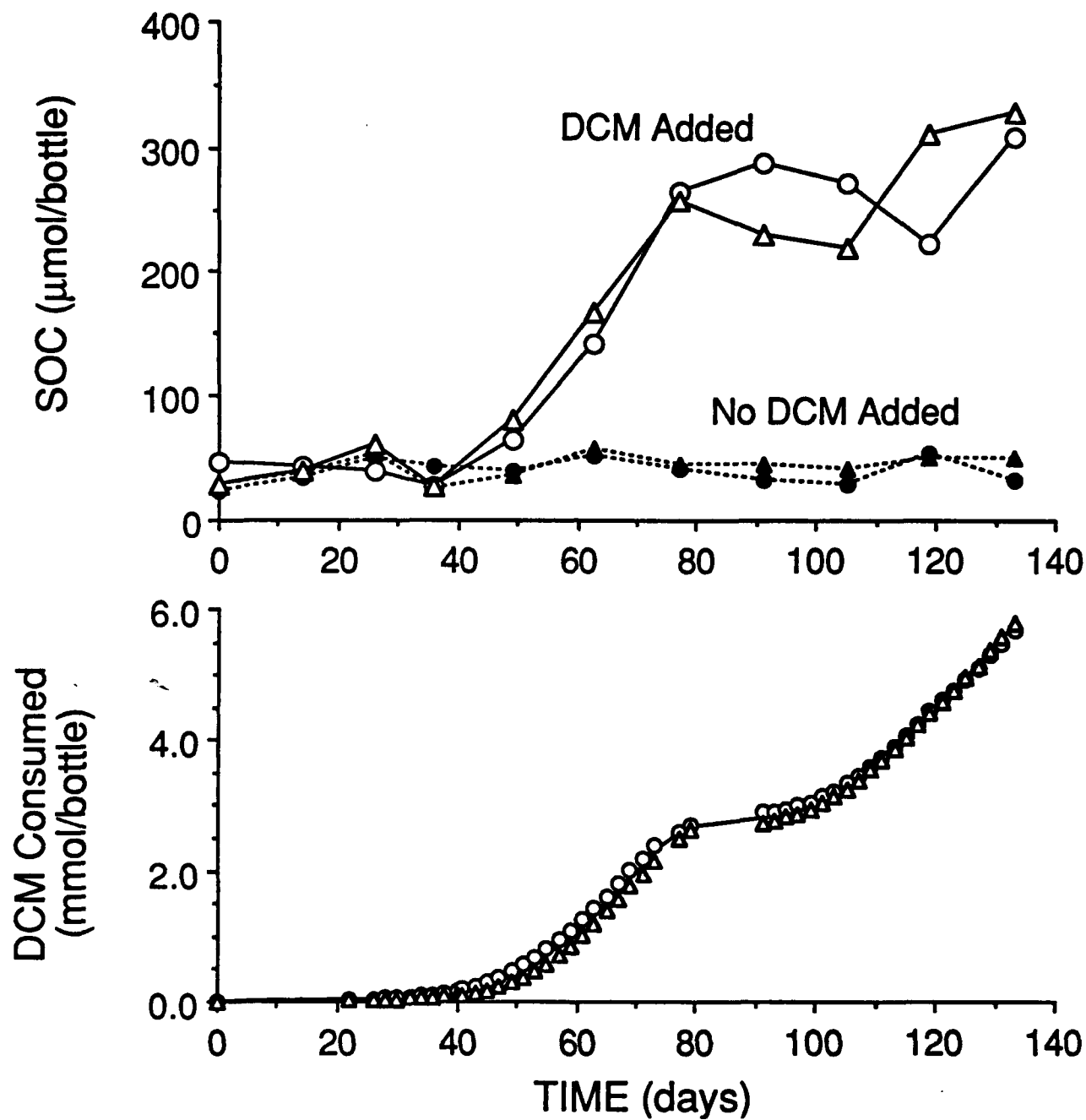


FIGURE 6



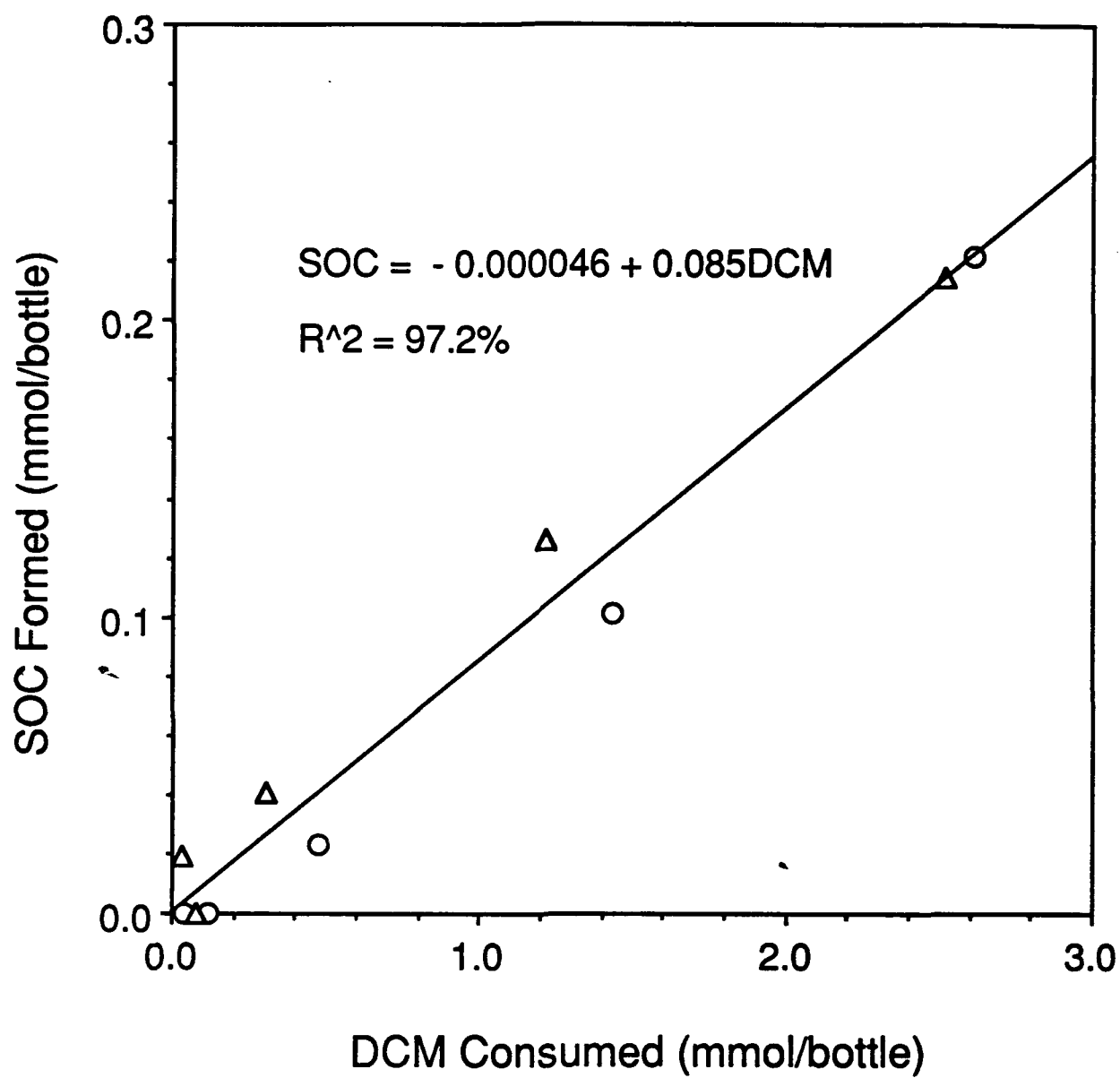


FIGURE 7

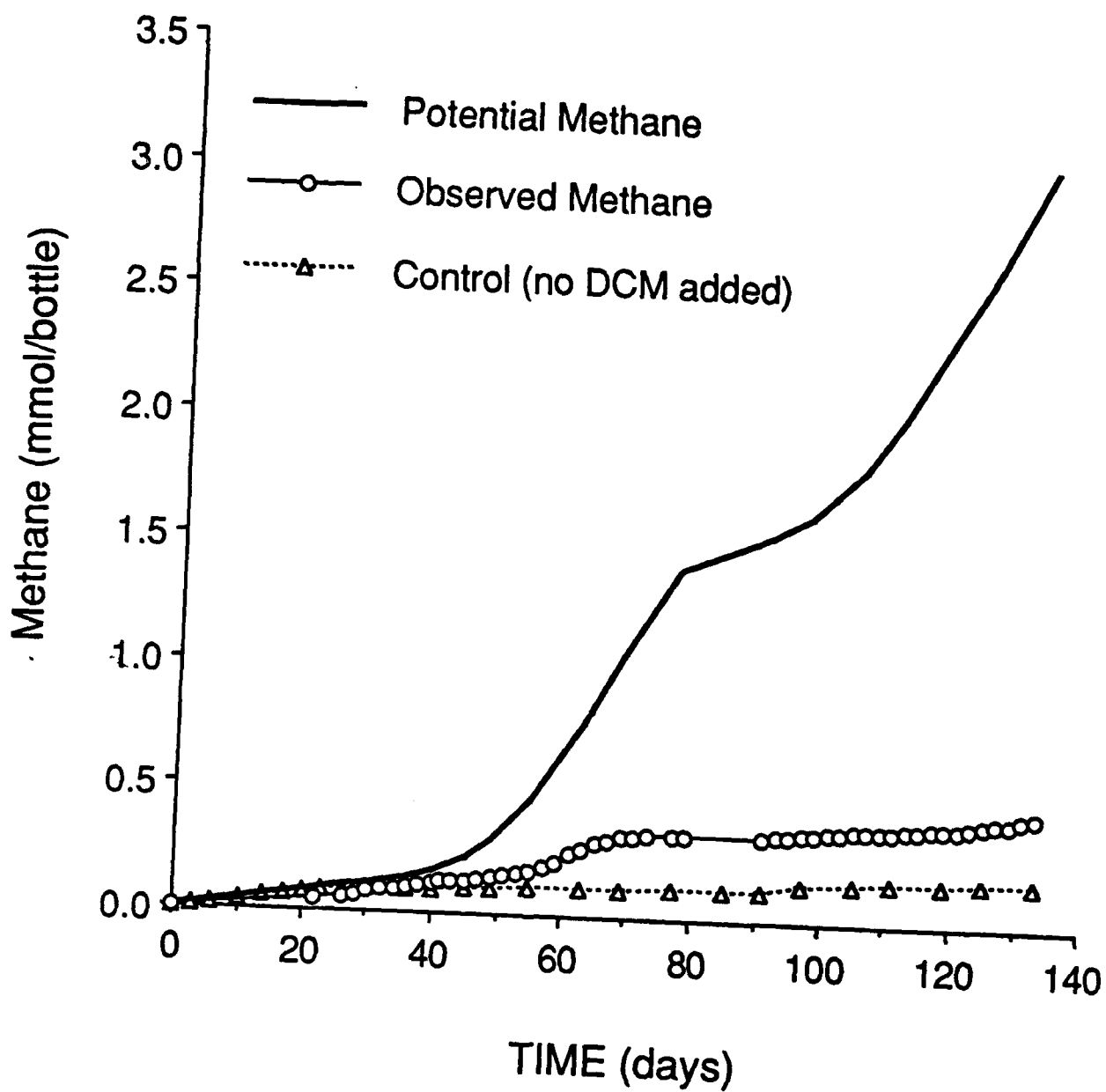


FIGURE 8

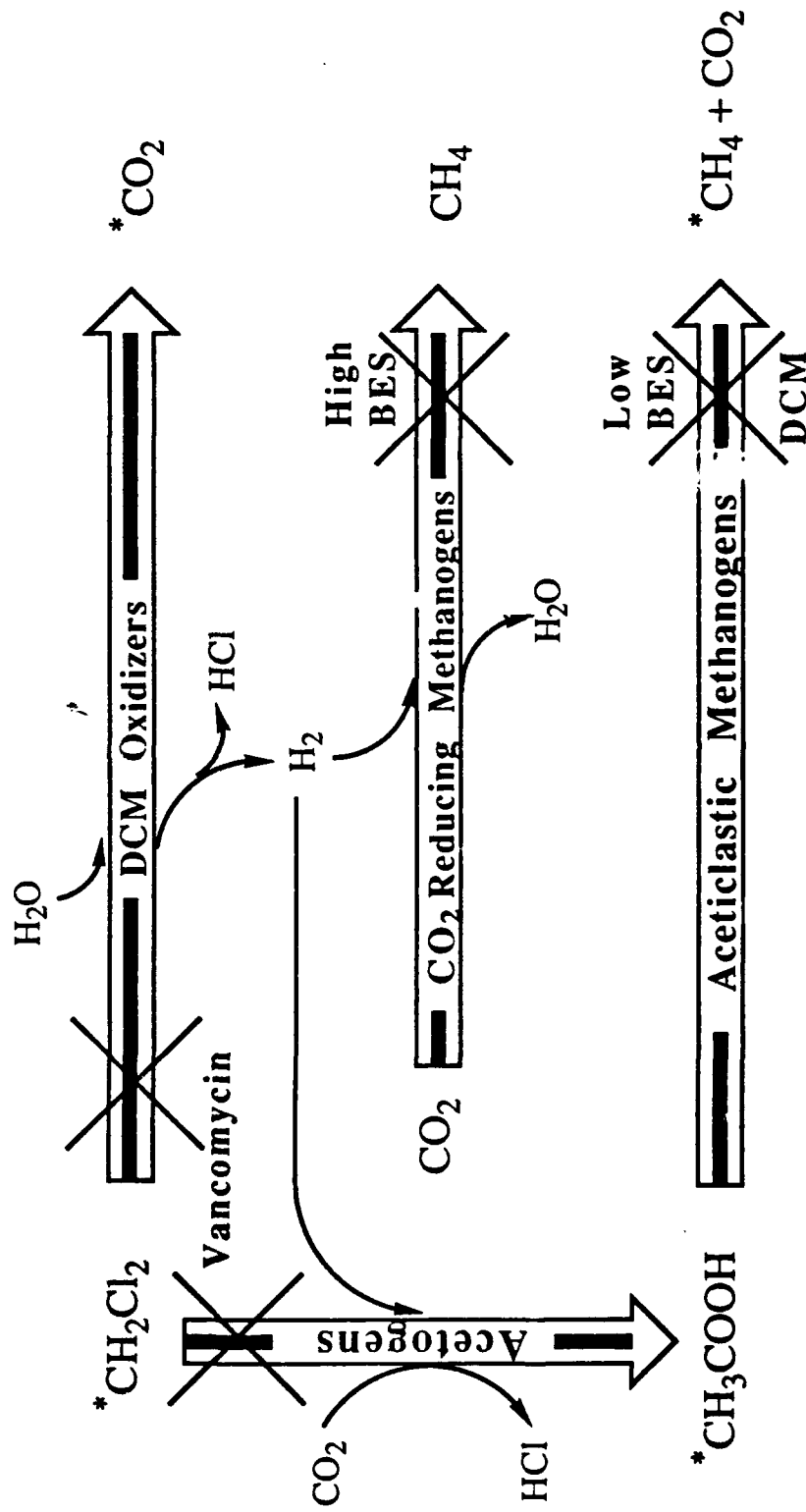


FIGURE 9

D. L. Freedman and J. M. Gossett

# BIODEGRADATION OF DICHLOROMETHANE IN A FIXED-FILM REACTOR UNDER METHANOGENIC CONDITIONS

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## INTRODUCTION

Since 1972, U. S. production of dichloromethane (DCM) has ranged from 212 to 287 million kilograms per year (U. S. ITC 1970-1985). Principal applications include paint removal and stripping, use in aerosols (*e.g.*, for spray paints and lubricants), use in manufacture of polycarbonate resin for the production of thermoplastics, and metal cleaning and degreasing. A consequence of this widespread use has been the appearance of DCM in groundwater systems. Concern over the presence of DCM in drinking water supplies is based on relatively recent studies which indicate the compound causes lung and liver cancer in mice (*Chemical Week* 1988). Although not the most widely occurring groundwater contaminant (trichloroethylene is), DCM is among the more difficult chlorinated aliphatics to remove by conventional remediation techniques (such as air stripping) because of its comparatively low Henry's constant (Gossett 1987). Interest in biological processes for remediation has grown recently because these offer the prospect of converting DCM to environmentally acceptable products.

Work we recently conducted with DCM using suspended growth cultures focused on the fundamental factors affecting its biodegradation under methanogenic conditions. Enrichment cultures were developed (in 160 mL serum bottles, maintained at 35 C) that used DCM as a sole organic carbon and electron donor source. A model for DCM biodegradation was proposed, indicating that the two principal modes of transformation were oxidation to CO<sub>2</sub> and fermentation to acetic acid (HAc). Thus, DCM degradation was a disproportionation, since oxidation of DCM made reducing equivalents available for reduction of an equal amount of DCM. The organism mediating DCM biodegradation was most likely an acetogen. Methanogenic bacteria in the mixed culture then used the products of acetogenic DCM degradation to form methane. CO<sub>2</sub>-reducing methanogens used reducing equivalents gained from DCM oxidation to form methane; at least a portion of the reducing equivalents were available as hydrogen. Acetoclastic methanogens produced methane from the HAc formed by acetogenic fermentation of DCM. Assuming acetogenic bacteria did mediate DCM degradation, it is likely that the recently elucidated acetyl-CoA

pathway was involved (Ljungdahl 1986). Since acetyl-CoA is a key intermediate in this pathway, it becomes apparent how DCM can serve as a growth substrate (Freedman & Gossett 1991).

Environmental conditions in DCM-contaminated groundwater are quite different from those employed in laboratory studies. First, attached growth conditions — rather than suspended growth conditions — will prevail, whether the mediating organisms are employed *in situ* or in an engineered, on-site, fixed-film reactor. Second, the operating temperature will be considerably lower than the optimum mesophilic one of 35 C. Pilot studies with a fixed-film reactor system were therefore undertaken to assess the applicability of laboratory results to conditions more like those expected during actual remediation.

The specific objectives of this study were: (1) to determine if DCM degradation can be sustained under attached growth conditions, at hydraulic residence times (HRT) ranging from 0.25 to 2 days; (2) to determine if DCM degradation can be sustained at 20 C, when DCM serves as the major organic carbon and electron donor source; (3) to examine the level of micronutrients — provided as yeast extract (YE) — needed in the system feed; (4) to determine the highest influent concentration of DCM that can be degraded at a 2-day hydraulic retention time (HRT); and (5) to analyze the fate of DCM during biodegradation in the fixed-film reactor system, using [ $^{14}\text{C}$ ]DCM.

## MATERIALS AND METHODS

### System Description

The treatment system, located in a constant temperature room, consisted of two columns in series (Figure 1). A precolumn was used solely to reduce the redox potential of the feed to the treatment column, thereby ensuring that methanogenic conditions prevailed in the treatment column. Both columns were constructed from conical glass pipe (51 mm i.d. x 60 mm o.d. x 914 mm; Corning), filled to capacity with 6 mm glass beads. A peristaltic pump (Cole-Parmer) delivered refrigerated influent through the system. All components in the treatment column

consisted of either glass, stainless steel, or Teflon™. A valve was located in the tubing coming from the precolumn, making it possible to seal off of the treatment column (as was necessary when changing sampling port septa; see below). DCM-saturated water was added via a syringe pump (Sage Instruments); Pressure-Lok™ gas syringes (Supelco, Inc.) were used. A valve in the DCM addition tubing made it possible to shut off the syringe when refilling it. The influent sampling port consisted of a tee modified to hold an 11-mm Teflon™-faced rubber septum (Supelco, Inc.) in an ULTRA-TORR™ Adapter (Cajon Co.).

Five sampling ports were located in the treatment column, spaced every 152 mm from the base of the column. Each port was constructed from a threaded glass connector tube (Ace Glass); inside the connector was a custom-fabricated stainless steel sampling tube that extended into the center of the treatment column to ensure that wall effects were minimized during sampling. The outside end of the sampling tube was machined to accept a Teflon™-faced rubber septum (11 mm) and aluminum crimp cap (Supelco, Inc.). A Teflon™ bushing and O-ring secured the sampling tube inside the connector, and made a leak-tight seal.

The top of the column consisted of a glass cap that contained the final effluent sampling port, constructed as above, and located 200 mm above the fifth port on the treatment column. A glass outlet tube on the cap was fitted with a stopcock so that the effluent end of the treatment column could be sealed off. Tubing from the stopcock was connected to a U-tube. The discharge side of the U-tube consisted of a 250-mL aspirator bottle that provided a reservoir of liquid to flow back into the column when samples were removed, thereby preventing the entry of air. A glass tee at the top of the aspirator bottle was open to the atmosphere and therefore served as a siphon break. The void volumes of the precolumn and treatment column were 660 mL and 730 mL, respectively.

When the sampling port septa were punctured several times (usually 5 to 10) they were replaced to prevent leakage and the introduction of air. The treatment column was placed on its side and, as a stream of  $N_2/CO_2$  was placed close to a septum, the crimp cap was removed, and a new septum and cap were quickly slid into position as the old septum was pushed away.

Table 1 lists the basal salts medium used in the system feed. It was prepared in 15-L batches and refrigerated. The feed was not augmented with HAC and YE until just prior to its use, to reduce the possibility of growth in the feed reservoir. HAC (present at 100 mg/L) provided the electron donor needed to consume oxygen and lower the redox potential in the precolumn; YE provided micronutrients. No reductant ( $\text{Na}_2\text{S}$ ) was used, since the precolumn accomplished this task.

### Analytical Methods

The concentrations of DCM and methane in the treatment column were determined by withdrawing 4-mL samples from each port, beginning with the effluent end and progressing sequentially to the influent port. A 5-mL Pressure-Lok™ syringe (Series C; Supelco, Inc.) and specially fabricated 102-mm side port needle (Dynatech Precision Sampling Corp.) were used. The syringe and needle were flushed with  $\text{N}_2/\text{CO}_2$ , in a 70:30 ratio, prior to puncturing the sampling port septum. Immediately after removing 4 mL, it was injected into a 15-mL serum bottle stoppered with a slotted grey-butyl septum and aluminum crimp cap. The seven samples were placed in a 35 C reciprocating water bath, where DCM and methane in the serum bottles equilibrated between the aqueous and gaseous phases. A 0.5-mL headspace sample was then injected into to a Perkin-Elmer (Model 8500) gas chromatograph (GC), equipped with a flame ionization detector and a 3.2-mm x 2.44-m stainless steel column packed with 1 percent SP-1000 on 60/80 Carbowax-B (Supelco, Inc.), as previously described (Freedman & Gossett 1989).

The GC response factor for DCM was determined by adding 4 mL of water to replicate 15-mL serum bottles. A known amount of DCM (about 2.3  $\mu\text{moles}$ ) was added to the bottles. Once equilibrated at 35 C, a 0.5-mL headspace sample was injected into the GC, as above. DCM additions (determined gravimetrically) were made from a stock solution containing DCM dissolved in methanol; the final concentration of methanol in the serum bottles was about 0.5 percent (v/v), well below the level that affects DCM's Henry's constant (Gossett 1987). The detection limit for



DCM was approximately  $0.63 \mu\text{M}$  ( $53 \mu\text{g/L}$ ). For  $\text{CH}_4$ ,  $0.15 \text{ mL}$  of pure gas was injected into replicate serum bottles containing  $4 \text{ mL}$  of water. The bottles were equilibrated at  $35 \text{ C}$ , and then a  $0.5\text{-mL}$  headspace sample was injected into the GC. The coefficients of variation for the GC response factors were 1.89 percent for DCM, 1.21 percent for  $\text{CH}_4$ .

The concentration of DCM in the DCM-saturated water added to the treatment column was determined by injecting  $50 \mu\text{L}$  into a  $160\text{-mL}$  serum bottle filled with  $100\text{-mL}$  of distilled, deionized water. Once the serum bottle was equilibrated at  $35 \text{ C}$ , a  $0.5\text{-mL}$  headspace sample was analyzed by GC, as described previously (Freedman & Gossett 1989). Based on 41 samples, the DCM-saturated water contained an average concentration of  $0.225 \mu\text{mole DCM}/\mu\text{L}$  (coefficient of variation = 1.04%). Loss of DCM from the Pressure-Lok™ delivery syringe while it infused DCM-saturated water to the treatment column influent flow was minimal. The concentration of DCM in the DCM-saturated water after it had been in the syringe for at least 2 days (the average time between filling) was  $0.218 \mu\text{mole DCM}/\mu\text{L}$  ( $n = 41$ ; coefficient of variation = 5.08%), or a decrease of only 3.1 percent.

### **[ $^{14}\text{C}$ ]DCM Studies**

The final operational phase of the study involved addition of [ $^{14}\text{C}$ ]DCM along with DCM-saturated water at a 0.25-day HRT. A solution was prepared by combining approximately  $11 \text{ mL}$  of DCM-saturated water and  $1.2 \text{ mL}$  of [ $^{14}\text{C}$ ]DCM stock (containing approximately  $2 \times 10^7 \text{ dpm/mL}$ ), resulting in  $1970 \text{ dpm}/\mu\text{L}$  of solution. Analysis of the [ $^{14}\text{C}$ ]DCM stock (used in the  $35 \text{ C}$  suspended growth studies) indicated 97 percent of the label consisted of DCM (Freedman & Gossett 1991). Based on an average delivery syringe flow rate of  $50.8 \mu\text{L/hr}$  and an average treatment column flow rate of  $118 \text{ mL/hr}$ , the expected level of radioactivity in the treatment column was  $848 \text{ dpm/mL}$ . The DCM/[ $^{14}\text{C}$ ]DCM solution contained  $0.145 \mu\text{mole DCM}/\mu\text{L}$ ; this was less than the routine saturated solution because the [ $^{14}\text{C}$ ]DCM stock used in the mixture was less than saturated.

When the concentration of total  $^{14}\text{C}$  activity in the treatment column effluent was within 10 percent of the expected level in three consecutive tests, 4-mL samples were withdrawn from each of the treatment column ports and addition of [ $^{14}\text{C}$ ]DCM was stopped. Total DCM and  $\text{CH}_4$  levels were determined by headspace GC analysis (0.5 mL sample), as described above. Then the distribution of  $^{14}\text{C}$  was determined; the first step was to raise the sample pH above 10 (to force all  $\text{CO}_2$  into the aqueous phase of the serum bottles) by adding 5  $\mu\text{L}$  of 8 M NaOH. Levels of dpm in each serum bottle were then measured by adding the following subsamples to 15 mL of liquid scintillation cocktail: (A) 0.5-mL headspace sample from the serum bottle, injected into a liquid scintillation vial modified to hold a septum in the cap, as previously described (Freedman & Gossett 1989); (B) one mL of basified sample; (C) one mL of basified sample that had been purged for at least 10 min with  $\text{N}_2$  ( $>50$  mL/min); and (D) one mL of acidified sample (approximately 10  $\mu\text{L}$  of HCl added to the basified sample) that had been purged for at least 10 min with  $\text{N}_2$  ( $>50$  mL/min).

Total dpm in the serum bottle was the sum of the headspace (A) plus basified sample counts (B); however, only the headspace sample from the influent port was significantly different from a blank. The fraction purgeable at high pH was  $A + B - C$ ; this was presumably either DCM or  $\text{CH}_4$ . The fraction purgeable only at low pH was  $C - D$ ; this was presumably  $\text{CO}_2$ . Compounds not purgeable at low pH (D) were identified as nonstrippable residue (NSR).

$^{14}\text{C}$  activity was assayed with a model 9800 liquid scintillation counter (Beckman Instruments), following addition of 15 mL of liquid scintillation cocktail (Fisher Scientific) to each one-mL sample. Corrections for counting efficiency were made according to a quench curve (sample H# versus efficiency).

## RESULTS

### Initial Testing with Water and DCM Only

Before starting the DCM degradation studies, the treatment column was operated at a 2-day HRT with only distilled, deionized water and DCM to evaluate abiotic losses. Results are shown in Figure 2. For the first 10 days, the DCM level in the column was well below the targeted concentration of 100  $\mu\text{M}$ . On day 10, a length of Viton™ tubing used to connect the DCM-saturated water syringe needle to the treatment column influent tubing was replaced with stainless steel tubing (1.59 mm), and all Viton™ O-rings were replaced with Teflon™ ones. Shortly thereafter, the concentration of DCM in the influent and effluent reasonably approached the target level, and remained so for an extended period of time. Although Viton™ is chemically resistant to DCM, it appears that it is also quite permeable with respect to DCM. The results from Figure 2 indicated that disappearance of DCM from the treatment column, once inoculated, was a result of biodegradation, not sorptive or diffusive losses.

As shown in Figure 2, the observed influent concentration of DCM varied considerably more than the effluent level. A likely reason was the poor degree of mixing between the DCM-saturated water and treatment column bulk flow. Even in the 3.18-mm tubing prior to the influent sampling port, where the velocity was highest, laminar flow conditions prevailed (Reynolds number = 28, based on  $Q = 5.47 \times 10^{-8} \text{ m}^3/\text{s}$ , i. d. =  $2.10 \times 10^{-3} \text{ m}$ , and  $v = 7.27 \times 10^{-7} \text{ m}^2/\text{s}$ ). For this reason, the influent DCM levels shown in all subsequent figures have been calculated, using measured values for the treatment column flow rate, the syringe pump flow rate, and the concentration of DCM in the DCM-saturated water.

**Inoculation of the Precolumn and Treatment Column**

Before operating the two columns in series as a single system, each was inoculated and operated independently to demonstrate proper performance of its designated task, i.e., for the precolumn to reduce the redox potential of the system feed and for the treatment column to degrade DCM. All of these initial operations were conducted at 35 C.

The precolumn was inoculated on April 6, 1989; the inoculum consisted of 2 L of basal salts (in the same proportions listed in Table 1), 336 mg of HAc, and 0.5 L of mixed liquid from a 15-L laboratory digester, described previously (Freedman & Gossett 1989). No special precautions were taken to make the inoculum anoxic, since the organisms present created such conditions once in the column. For the first five days, the precolumn was operated in a recirculation mode; continuous flow was then started at a 4-day HRT. Approximately one month later, this was reduced to a 2-day HRT, prior to introducing flow to the treatment column. HPLC/refractive index analysis (described in Freedman & Gossett 1991) of several effluent samples indicated HAc levels were consistently below the detection limit (about 5 mg/L).

The inoculum source for the treatment column was a 1.37-L culture, contained in a 2.20-L glass bottle; a stir bar was used to keep the contents agitated. This culture readily degraded approximately 120- $\mu$ M additions of DCM every other day and produced nearly stoichiometric amounts of  $\text{CH}_4$ . The treatment column was inoculated on April 25 and operated in recirculation mode; the starting concentration of DCM was approximately 120  $\mu$ M. Samples were taken periodically to monitor the rate of DCM degradation. Because the rate was initially well below what had been observed in the inoculum source, additional inoculum was added (5 mL at each sampling port) on May 3. Once a satisfactory rate of DCM degradation was subsequently established, the precolumn and treatment column were connected in series, at a 2-day HRT, on May 17.

## DCM Degradation Profiles and Continuous Flow Operation

Once the precolumn and treatment column were connected in series on May 17, continuous flow operation was started using the system feed described in Table 1, plus 100 mg/L HAc and 50 mg/L of YE. Table 2 lists the changes in operational parameters over time, including temperature, YE concentration in the system feed, DCM influent concentration, and HRT.

**Operation at a 2-Day HRT.** During the first phase of operation (from May 17 to June 8), the columns were operated at 35 C with 50 mg/L of YE in the system feed, a DCM influent concentration of approximately 100  $\mu\text{M}$ , and a 2-day HRT. Samples taken on three occasions indicated nearly 99 percent of the DCM was degraded by port 1, with no detectable amount present by port 2. A sample profile (for June 2) is given in Figure 3. Methane output rose in accordance with the disappearance of DCM, but the amount produced was well above what could be explained by stoichiometric conversion of DCM (i.e., 0.5 mole  $\text{CH}_4$ /mole DCM degraded). The large amount of YE in the system feed most likely contributed to this higher than expected methane yield from the treatment column.

Having demonstrated DCM degradation at 35 C, the temperature was gradually lowered to 20 C over a 14-day period, holding all other conditions the same. The lower temperature did not affect the ability of the treatment system to biodegrade DCM, although complete conversion was not observed until port 3. Total methane output was similar, but occurred more gradually along the length of the column.

The next phase of operation (July 3 to 19) involved reducing the amount of YE in the system feed from 50 to 5 mg/L. Two sets of column samples taken during this interval indicated no affect on the ability of the treatment column to degrade DCM. Methane output gradually declined to about 290  $\mu\text{M}$ .

YE in the system feed was then lowered to the target level of 0.5 mg/L and was kept there for all subsequent studies. Between July 19 and October 4, the system was operated at 20 C with

a DCM influent concentration of 100  $\mu\text{M}$ , 0.5 mg/L YE, and a 2-day HRT. Figure 4 presents average results for this period. The DCM degradation profile appeared similar to what was observed at 35 C and a higher level of YE; i.e., essentially all of the DCM was degraded in the first 152 mm of the column. However, methane output was lower, and significant amounts were not observed until the fifth port and the effluent port. One reason for this may have been the increased sensitivity of methanogens to DCM at 20 C compared to 35 C.

The next phases of system operation involved step increases in the DCM influent concentration; the aim was to determine how high a level could be reached before breakthrough and/or failure occurred. The system maintained its ability to degrade DCM when the influent concentration was raised to approximately 150  $\mu\text{M}$ , then 250  $\mu\text{M}$ , then 300  $\mu\text{M}$ , then 400  $\mu\text{M}$ , and then 530  $\mu\text{M}$ . Breakthrough did not occur until the average DCM influent concentration was stepped up to approximately 780  $\mu\text{M}$ . Three sets of samples were taken at this level, and the effluent DCM concentration increased with each analysis. Concurrent with the appearance of DCM in the effluent was a sharp reduction in total methane output, to only 85  $\mu\text{M}$  on January 5, 1990 (Figure 5); breakthrough of DCM inhibited methanogenesis at all levels of the treatment column.

In an effort to reestablish complete DCM degradation, the influent concentration was reduced to about 315  $\mu\text{M}$  beginning on January 5. The profile obtained was very similar to what had been observed when the same level of DCM was added as when the DCM influent concentration was being stepped up. However, methane output remained quite low (approximately 100  $\mu\text{M}$ ), even though DCM no longer appeared in the upper reaches of the column.

The influent concentration of DCM was boosted again to approximately 460  $\mu\text{M}$ . DCM disappeared below the detection limit even sooner than at a similar influent DCM level used previously, but methane output (about 100  $\mu\text{M}$ ) was nearly seven times less. Thus, even though nearly three months had past since the influent DCM was lowered below the breakthrough concentration (on January 5; see Table 2), methanogenesis did not recover to the level observed prior to breakthrough of DCM.

**Operation at <2-day HRT.** Having established the highest DCM concentration that could be degraded (approximately 530  $\mu\text{M}$ ) — at a 2-day HRT, 20 C, and 0.5 mg/L of YE in the system feed — before reaching breakthrough, the next phase of operation involved lowering the HRT to one day. This was done over a 6-day interval, beginning on March 21, 1990. Three sets of column samples were taken once stable operation at this HRT was demonstrated. At an influent DCM level of 230  $\mu\text{M}$ , most of the DCM was consumed by port 1, and all of it was degraded below the detection limit by port 2, even at the lower HRT. Average methane output at a one-day HRT was only 29  $\mu\text{M}$ .

Over a 10-day interval, the treatment column HRT was lowered from one-day to 0.5-day, beginning on April 20. The column successfully degraded an influent DCM concentration of 190  $\mu\text{M}$ , but complete degradation was not achieved until port 5. Methane output (average = 18  $\mu\text{M}$ ) continued to fall in accordance with the lower influent DCM concentration and decrease in HRT.

The final phase of column operation involved lowering the HRT to 0.25-day, over a 6-day interval. At 130  $\mu\text{M}$  DCM and a 0.25-day HRT, DCM broke through the column, exiting at a concentration of 26  $\mu\text{M}$  ( $\text{CH}_4 = 7 \mu\text{M}$ ). When the DCM influent concentration was lowered to 90.6  $\mu\text{M}$  (on June 28), triplicate sampling events indicated degradation below the detection limit by port 4, as shown in Figure 6. It is unclear why the DCM concentration plateaued (at 10  $\mu\text{M}$ ) between ports 1 and 2. Average methane output was 11  $\mu\text{M}$ .

The analytical methods employed made it possible to detect chloromethane, if it appeared as a product of DCM biodegradation. However, chloromethane formation was never observed during any phase of the study.

The pH of the treatment column was monitored from the outset using the 4-mL samples from each port after they had been analyzed on the GC. Newly prepared system feed (after adding HAc) had a pH in the range of 6.8 to 7.4. In the treatment column, the pH ranged from 7.1 to 7.7. There tended to be a slight drop in pH from the influent port to all subsequent ports, indicating that most of the DCM was degraded in the first 152 mm of the column, thereby releasing the most amount of HCl.

## Fate of [ $^{14}\text{C}$ ]DCM

Having demonstrated stable system operation at a 0.25-day HRT, an experiment was performed to determine the fate of [ $^{14}\text{C}$ ]DCM. The HRT was maintained at 0.25-day, but the influent DCM concentration (beginning on July 21) fell from 90.6  $\mu\text{M}$  to 60.3  $\mu\text{M}$  since the DCM/[ $^{14}\text{C}$ ]DCM syringe solution contained a lower level of DCM. Results from the [ $^{14}\text{C}$ ]DCM study are summarized in Figure 7. The fraction purgeable at high pH (A+B-C) was presumed to be all DCM. Although it could have included methane, this possibility was unlikely because methane production in the treatment column was considerably inhibited: at the effluent port the methane level was 9.5  $\mu\text{M}$ , nearly 70 percent less than what could have been produced based on stoichiometric conversion of DCM (0.5 mole  $\text{CH}_4$ /mole DCM). Under similar conditions observed during serum bottle studies, none of the methane formed was labeled (Freedman & Gossett 1991).

Disappearance of [ $^{14}\text{C}$ ]DCM coincided with total DCM, based on GC analysis of sample bottle headspaces — i.e., essentially complete removal by port 4. Corresponding to the decrease in [ $^{14}\text{C}$ ]DCM along the length of the treatment column was an increase in  $^{14}\text{C}$ -labeled NSR and the fraction purgeable at low pH (C-D), presumed to be  $^{14}\text{CO}_2$ . At the effluent port, approximately 68 percent of the  $^{14}\text{C}$  activity was  $^{14}\text{CO}_2$  and 30 percent was  $^{14}\text{C}$ -labeled NSR.

The distribution of  $^{14}\text{C}$  activity in the  $^{14}\text{C}$ -labeled NSR fraction from the effluent port was evaluated by HPLC analysis, as previously described (Freedman & Gossett 1991). Approximately 85 percent of the  $^{14}\text{C}$ -labeled NSR was recovered as [ $^{14}\text{C}$ ]HAc.

Recovery of  $^{14}\text{C}$  activity was determined by comparing the measured dpm (A+B) at each port along the treatment column to the expected dpm (848 dpm/mL); on average, 85.1 percent was accounted for.



## DISCUSSION

Based on over 14 months of operating data, the results of this study indicate that DCM degradation can be sustained under methanogenic, fixed-film conditions, at a residence time as low as 0.25-day, DCM influent levels as high as 530  $\mu\text{M}$  (2-day HRT), 20 C, and a relatively low level of micronutrients (0.5 mg/L YE). These findings appear to be the first demonstration of a system capable of degrading DCM in a fixed-film reactor, under methanogenic conditions. Numerous other studies (Bouwer & McCarty 1982, 1983, 1985; Bouwer & Wright 1988; McCarty *et al.* 1984; Vogel & McCarty 1985, 1987) have previously demonstrated the capability of attached film reactors to degrade a variety of halogenated aliphatics — including tetrachloroethylene, trichloroethylene, carbon tetrachloride, and chloroform — under methanogenic conditions, but none of these evaluated biodegradation of DCM. The only attached-film research performed prior to this study with DCM was conducted under aerobic conditions (Galli & Leisinger 1985; LaPat-  
Polasko, *et al.* 1984; Rittmann & McCarty 1980).

An important finding from the serum bottle studies was the ability of the DCM-degrading organisms to use this compound as a sole organic carbon and electron donor source (Freedman & Gossett 1991). Based on this result, only DCM was added directly to the treatment column. It was possible, however, that some biodegradable organic matter other than DCM entered the treatment column via the precolumn effluent. A direct measure of organic carbon leaving the precolumn was not made. During the initial phase of operation there was evidence of carry over of organics from the precolumn (as a consequence of the large amount of YE initially present in the system feed), since more methane was produced than could be attributed to DCM degradation. However, the system operated successfully for more than 12 months with only 0.5 mg/L YE in the precolumn feed, after which methane output in the treatment column fell well below stoichiometric levels. A YE level lower than 0.5 mg/L may also be acceptable. Of the 100 mg/L of HAc added continuously to the precolumn, less than the detectable limit (about 5 mg/L) was observed in the treatment column influent. Methane was also at very low levels (typically below 3  $\mu\text{M}$ ) in samples

taken from the treatment column influent port. This suggests (but does not prove) that the HAc was consumed principally by organisms in the precolumn reducing the redox potential of the system, leaving little carry over of organics to the treatment column. A dense growth of biomass was observed in the first 100 mm of the precolumn. If the need for an auxiliary substrate to sustain DCM biodegradation is minimal or nonexistent, as suggested, considerable savings in operating costs will be realized during bioremediation (assuming methanogenic conditions already exist).

Another encouraging aspect of the fixed-film work was the ability of the treatment reactor to sustain DCM degradation at 20 C, a temperature approximating field conditions, rather than the 35 C environment used for the serum bottle experiments (Freedman & Gossett 1991). Whether or not even lower temperatures can be tolerated is yet to be determined.

At a 2-day HRT ( $Q = 365$  mL/day), a DCM influent concentration as high as  $530\text{ }\mu\text{M}$  ( $45\text{ mg/L}$ ) was degraded below the detection limit; this amounted to a DCM loading rate of  $8.1\text{ }\mu\text{mole/hr}$ . Because the syringe pump operated at discrete rates, the next DCM influent level tried was  $780\text{ }\mu\text{M}$  ( $66\text{ mg/L}$ ), at which point breakthrough occurred (DCM loading rate =  $11.9\text{ }\mu\text{mole/hr}$ ). Breakthrough was observed on one other occasion: at a 0.25-day HRT and a DCM influent concentration of  $130\text{ }\mu\text{M}$ , or  $11.0\text{ mg/L}$  (DCM loading rate =  $15.8\text{ }\mu\text{mole/hr}$ ). Once the influent concentration was lowered to  $90.6\text{ }\mu\text{M}$  ( $7.7\text{ mg/L}$ ), complete degradation was achieved; this amounted to a loading rate of  $11.0\text{ }\mu\text{mole DCM/hr}$ . Thus, the maximum DCM degradation rate at 20 C appeared to be in the range of 8 to  $11\text{ }\mu\text{mole/hr}$ . Successful biodegradation of DCM in the treatment column at a 0.25-day HRT was encouraging because operation at or below this level is essential in order to keep the size of an on-site, engineered reactor within economical limits.

The DCM-degrading organism(s) exhibited good resilience with regard to DCM overloading. As soon as the loading rate was reduced, the column regained its ability to completely degrade DCM. Methanogenesis was far less resilient; the breakthrough of DCM at an influent concentration of  $780\text{ }\mu\text{M}$  caused a reduction in methane output well below the level predicted based on stoichiometric conversion. This inhibition persisted long after the influent

DCM concentration was lowered below the breakthrough value. The observed inhibition of methanogenesis by DCM was consistent with our work that employed suspended growth cultures (Freedman & Gossett 1991).

Less than stoichiometric formation of methane raised the question of what happened to the biodegraded DCM. Results from the [ $^{14}\text{C}$ ]DCM experiment indicated that the principal products were (presumably)  $^{14}\text{CO}_2$  and [ $^{14}\text{C}$ ]HAc, both of which are environmentally acceptable. Our findings using DCM-degrading enrichment cultures in serum bottles (Freedman & Gossett 1991) showed that when methanogenesis was partially inhibited, the products of [ $^{14}\text{C}$ ]DCM degradation were approximately two-thirds  $^{14}\text{CO}_2$  and one-third  $^{14}\text{CH}_3\text{COOH}$ . Although  $^{14}\text{CH}_4$  formation was demonstrated when methanogenesis was uninhibited, none was produced when methanogenesis was partially inhibited. No attempt was made to measure  $^{14}\text{CH}_4$  during the [ $^{14}\text{C}$ ]DCM fixed-film reactor experiment; however, it seems likely that the same phenomenon occurred in the DCM treatment column when methanogenesis was partially inhibited.

Results from this study reinforce a conclusion of our work with suspended growth cultures (Freedman & Gossett 1991): to the extent that widespread use of halogenated aliphatic compounds continues, only those posing the least significant environmental hazard should be employed. Because DCM degrades to environmentally acceptable products under a variety of environmental conditions (including aerobic), it is one such compound that meets this criterion.

## **ACKNOWLEDGMENTS**

This research was supported by the U.S. Air Force Engineering and Services Center (AFESC), Tyndall AFB, FL, under contract no. F08635-86-C-0161, and by a post-doctoral fellowship from the Air Force Office of Scientific Research, administered by the American College of Toxicology.

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TABLE 1. Basal Medium Used for the System Feed.

Component	Quantity Per Liter of DDIW <sup>a</sup>
NH <sub>4</sub> Cl	200 mg
K <sub>2</sub> HPO <sub>4</sub> ·3H <sub>2</sub> O	101 mg
KH <sub>2</sub> PO <sub>4</sub>	55 mg
MgCl <sub>2</sub> ·6H <sub>2</sub> O	200 mg
Trace metal solution <sup>b</sup>	10 mL
NaHCO <sub>3</sub>	760 mg
Resazurin	1 mg

<sup>a</sup> Distilled, deionized water.

<sup>b</sup> 0.1 g/L MnCl<sub>2</sub>·4H<sub>2</sub>O; 0.17 g/L CoCl<sub>2</sub>·6H<sub>2</sub>O; 0.10 g/L ZnCl<sub>2</sub>; 0.20 g/L CaCl<sub>2</sub>; 0.019 g/L H<sub>3</sub>BO<sub>4</sub>; 0.05 g/L NiCl<sub>2</sub>·6H<sub>2</sub>O; 0.020 g/L Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, and 0.36 g/L FeCl<sub>2</sub>·4H<sub>2</sub>O.

TABLE 2. Summary of DCM Fixed-Film System Operating Parameters.

Dates of Operation		HRT	Temperature	Yeast Extract	Influent
From	To	(days)	(°C)	(mg/L)	DCM (μM)
5/17/89	6/8/89	2	35	50	100
6/8/89	6/22/89	2	35 → 20	50	100
6/22/89	7/3/89	2	20	50	100
7/3/89	7/19/89	2	20	5.0	100
7/19/89	10/4/89	2	20	0.5	100
10/4/89	10/11/89	2	20	0.5	150
10/11/89	10/16/89	2	20	0.5	250
10/16/89	10/25/89	2	20	0.5	300
10/25/89	11/2/89	2	20	0.5	400
11/2/89	11/8/89	2	20	0.5	530
11/8/89	1/5/90	2	20	0.5	780
1/5/90	1/30/90	2	20	0.5	315
1/30/90	3/21/90	2	20	0.5	460
3/21/90	3/27/90	2 → 1	20	0.5	-
3/27/90	4/20/90	1	20	0.5	230
4/20/90	4/30/90	1 → 0.5	20	0.5	-
4/30/90	6/17/90	0.5	20	0.5	190
6/17/90	6/23/90	0.5 → 0.25	20	0.5	-
6/23/90	6/28/90	0.25	20	0.5	130
6/28/90	7/21/90	0.25	20	0.5	90.6
7/21/90	7/27/90	0.25	20	0.5	60.3 <sup>a</sup>

<sup>a</sup> Includes [<sup>14</sup>C]DCM

**FIGURE LEGENDS**

Figure 1. Schematic diagram of the fixed-film reactor system. Yeast extract was added along with HAc to the system feed (to provide micronutrients), in amounts shown in Table 2.

Figure 2. DCM levels in the treatment column when only DCM and distilled, deionized water were added. 100  $\mu\text{M}$  was the DCM influent target concentration. Abiotic losses were minimal after replacing Viton™ tubing and O-rings on day 10.

Figure 3. Performance of the treatment column shortly after starting continuous flow operation.

Figure 4. Average performance of the treatment column when operated at a 2-day HRT. Error bars represent one standard deviation.

Figure 5. Breakthrough of DCM from the treatment column when added at an influent concentration of 780  $\mu\text{M}$ , equivalent to a DCM loading rate of 11.9  $\mu\text{mole/hr}$ .

Figure 6. Average performance of the treatment column when operated at a 0.25-day HRT. Error bars represent one standard deviation.

Figure 7. Distribution of  $^{14}\text{C}$  following addition of [ $^{14}\text{C}$ ]DCM to the treatment column, operated at a 0.25-day HRT, 20 C, and 60.3  $\mu\text{M}$  DCM in the influent. Fraction A + B - C was presumably DCM; fraction C - D was presumably  $\text{CO}_2$ ; fraction D was nonstrippable residue, most of which was HAc.

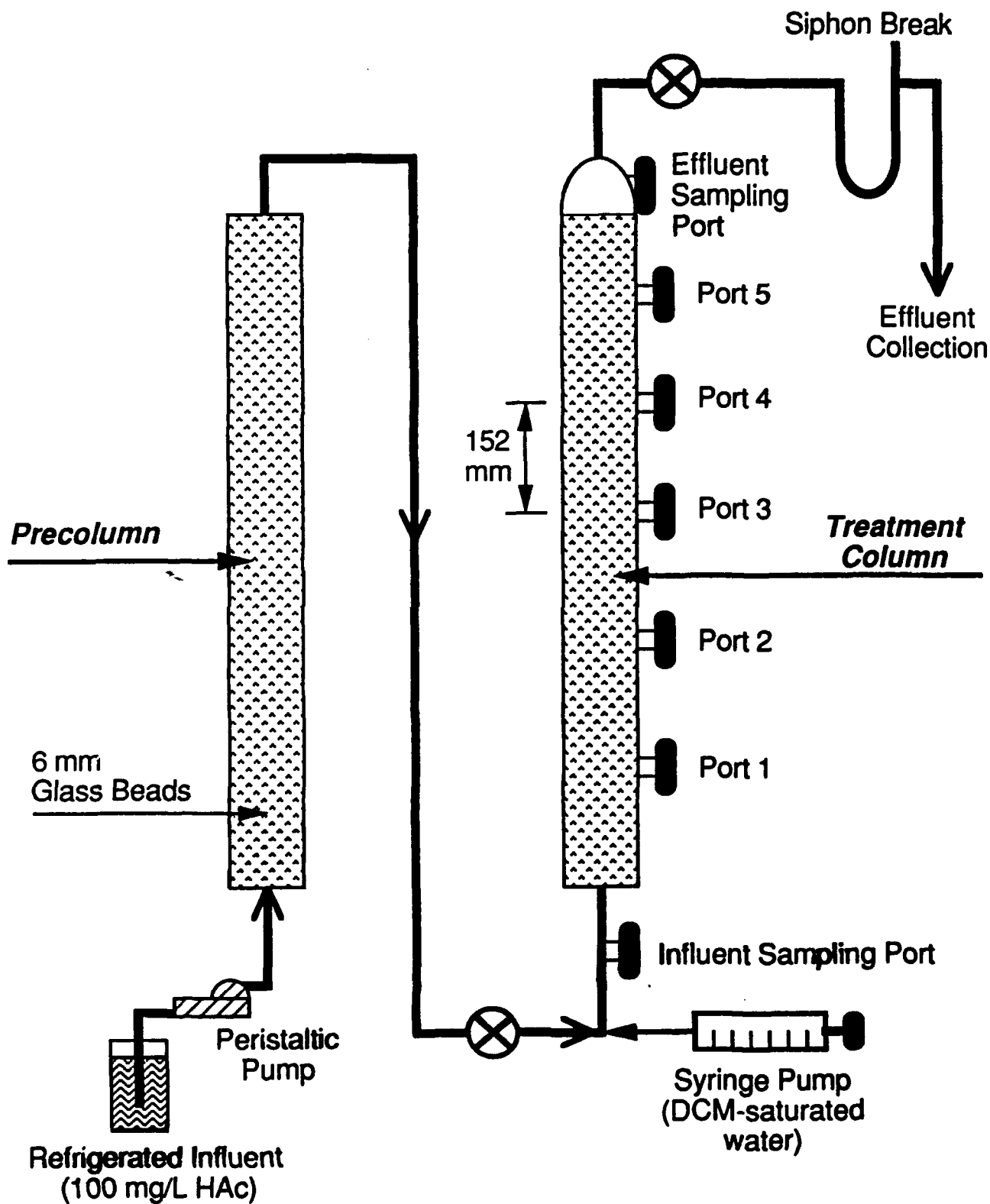


Figure 1, DCM Biodegradation in a Fixed-Film Reactor, 2nd submittal



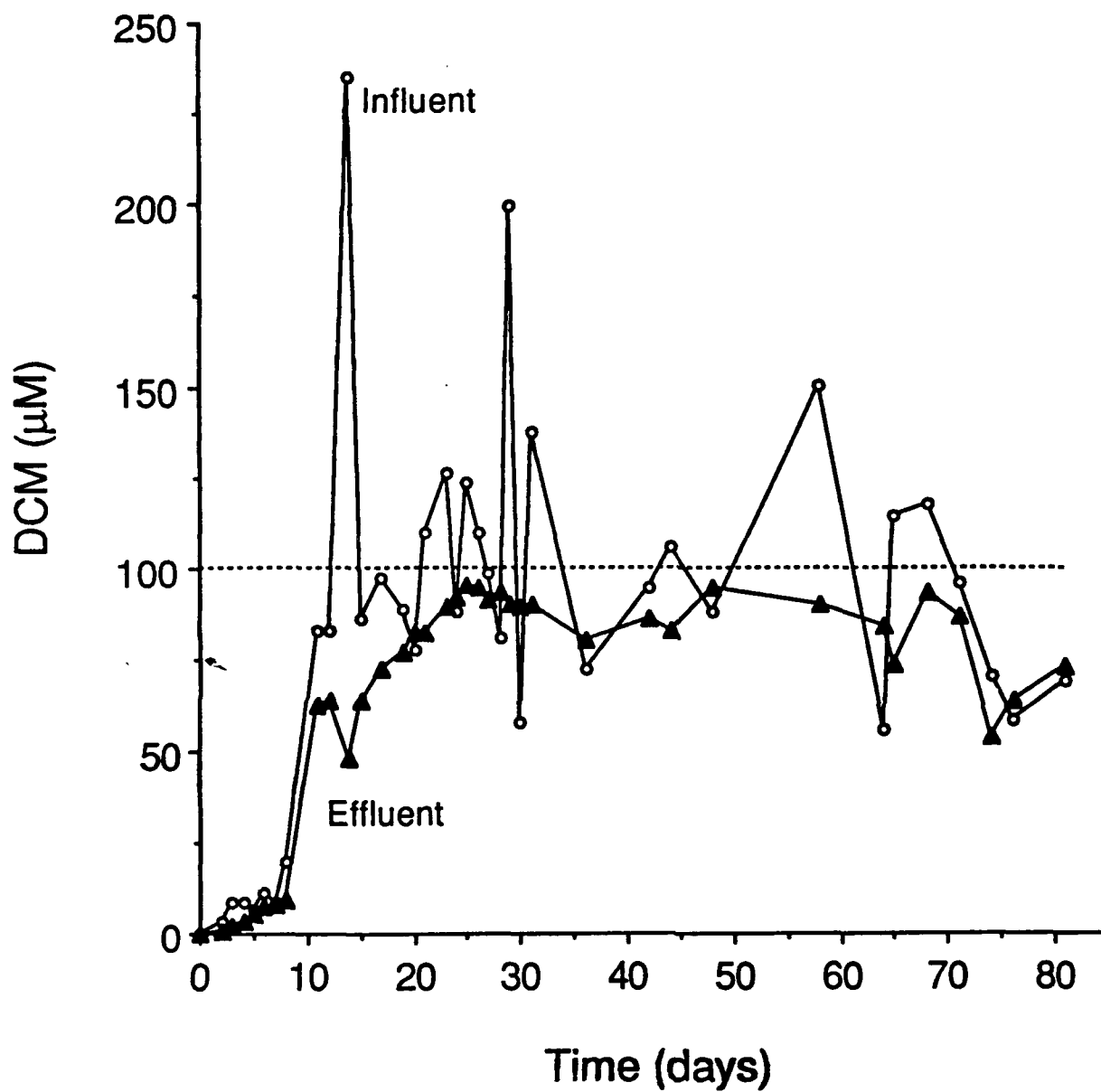


Figure 2, DCM Biodegradation in a Fixed-Film Reactor, 2nd submittal

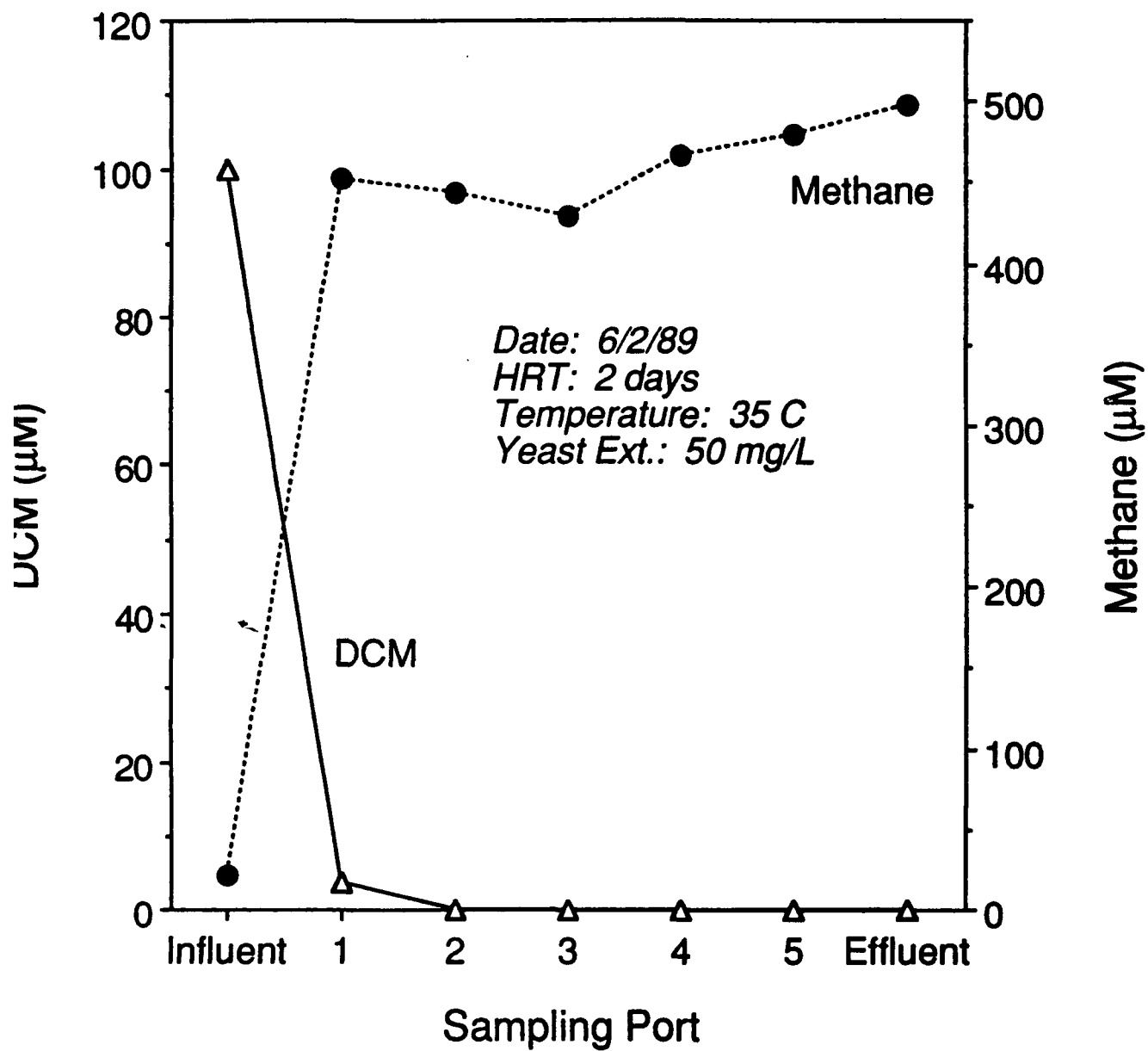


Figure 3, DCM Biodegradation in a Fixed-Film Reactor, 2nd submittal

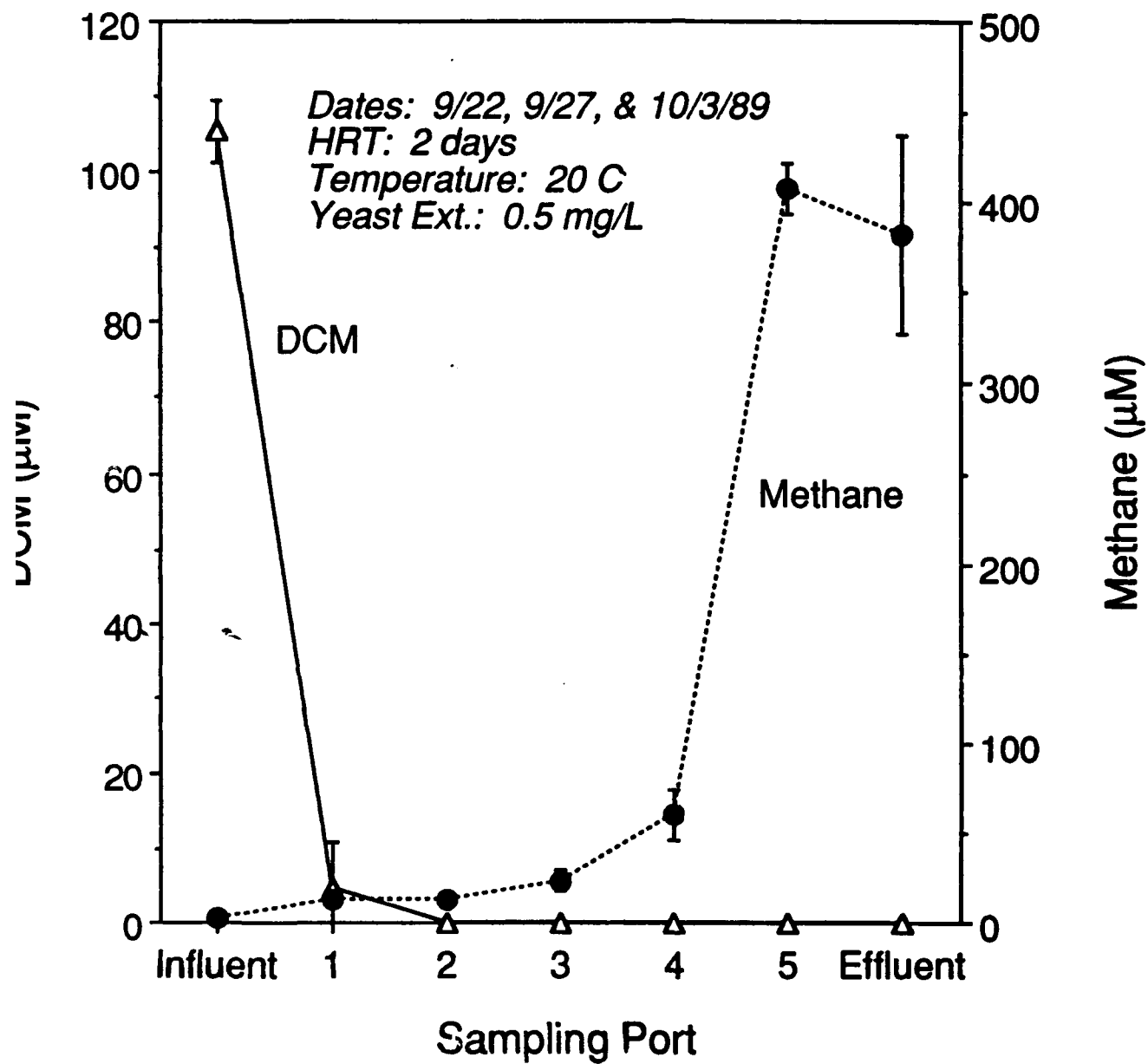


Figure 4, DCM Biodegradation in a Fixed-Film Reactor, 2nd submittal

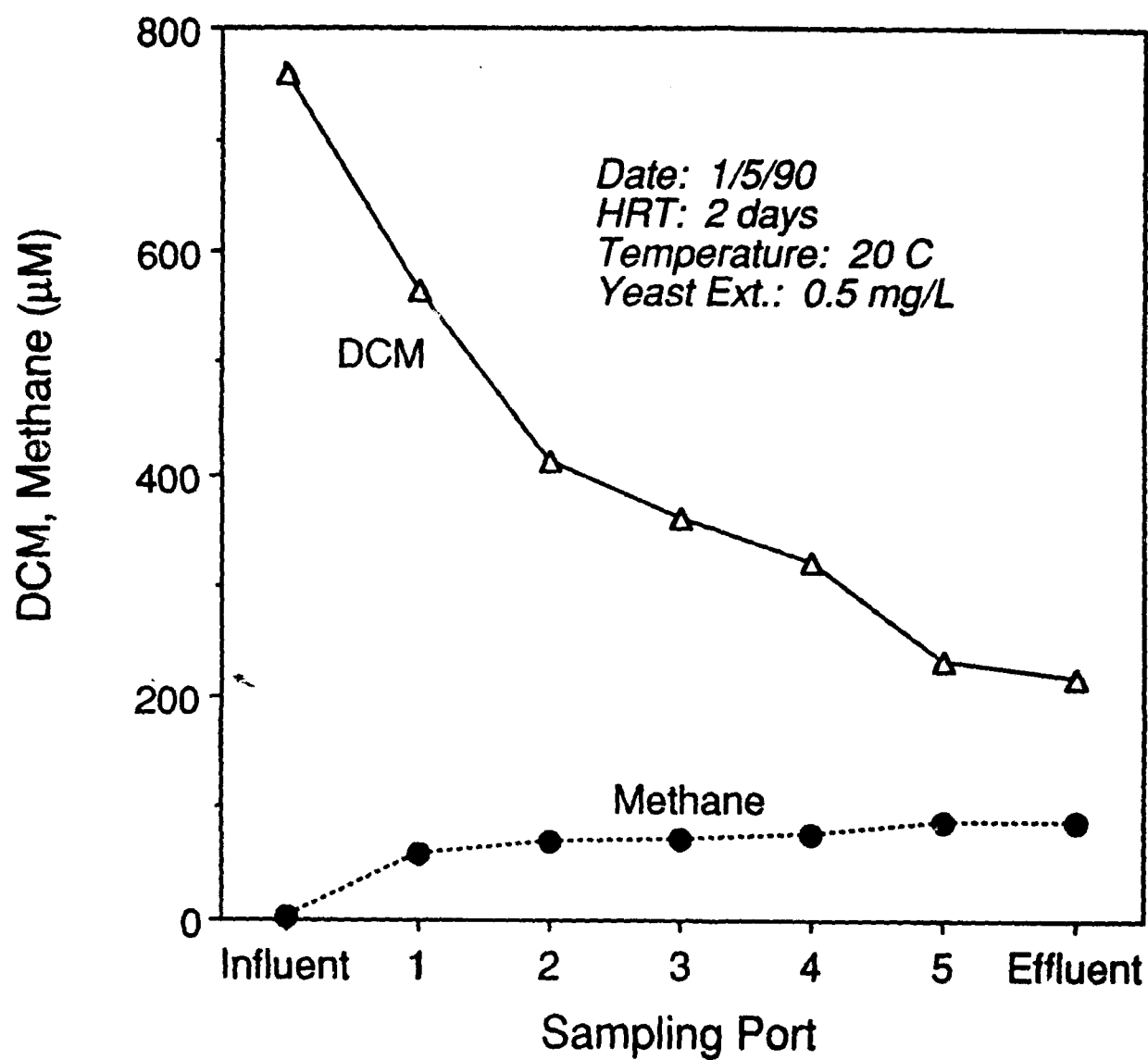


Figure 5, DCM Biodegradation in a Fixed-Film Reactor, 2nd submittal

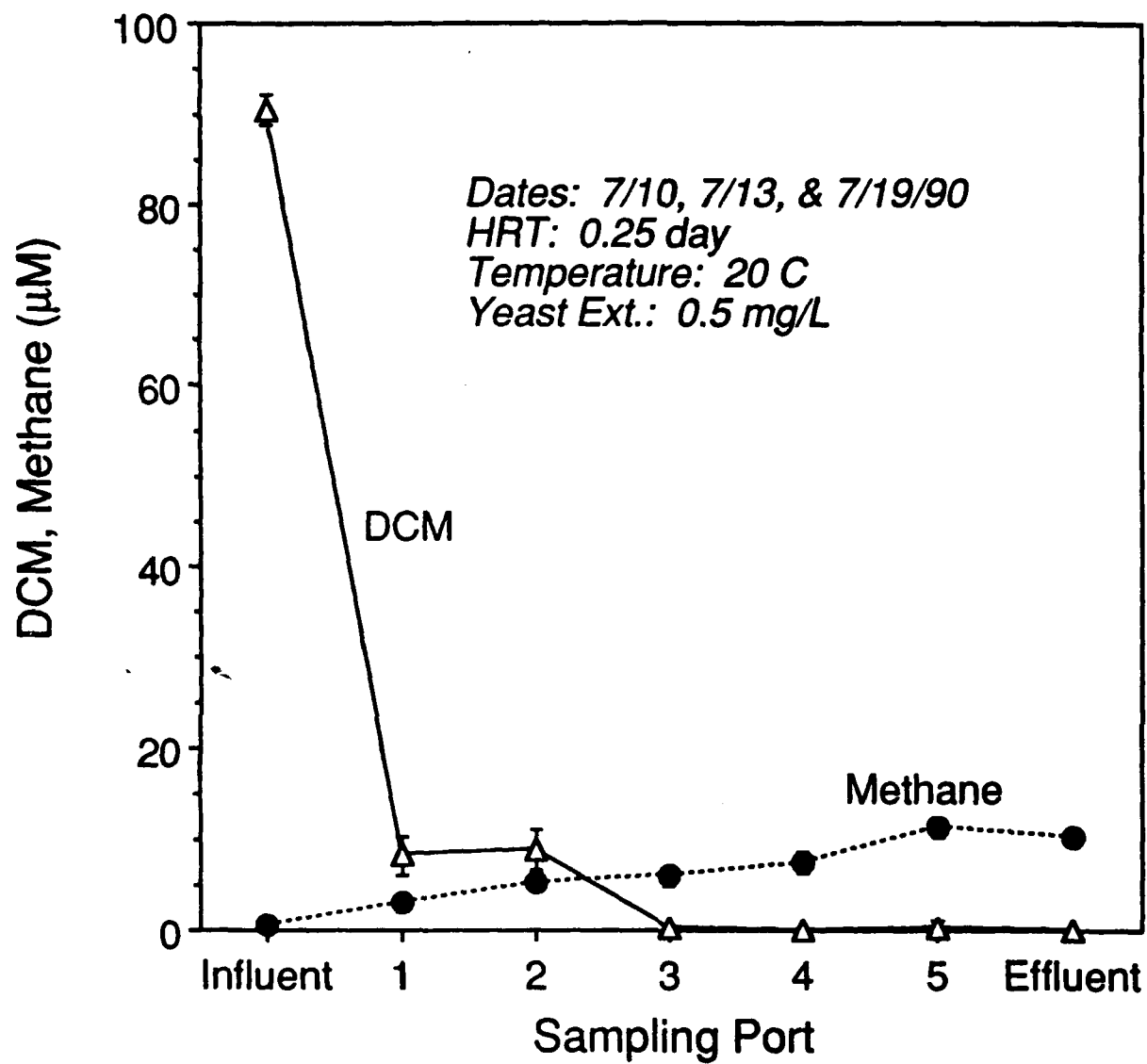
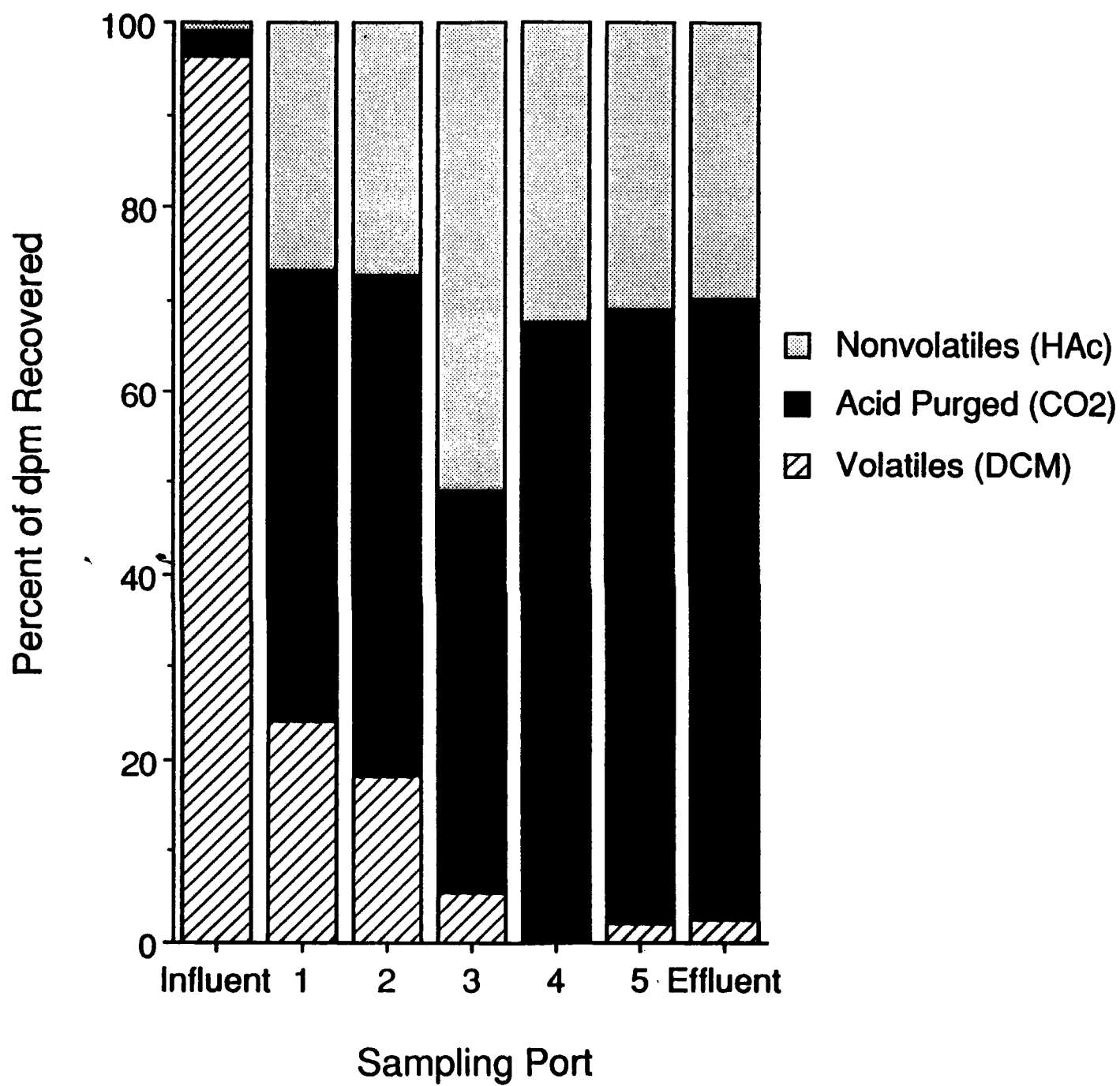


Figure 6, DCM Biodegradation in a Fixed-Film Reactor, 2nd submittal



**Figure 7, DCM Biodegradation in a Fixed-Film Reactor, 2nd submittal**